Creating greater value for biomass residues

Extraction of suberin and betulin using alternative solvents

Rui Manuel Cordeiro Ferreira

Dissertation presented to obtain the Ph.D degree in Engineering Sciences and Technology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

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Cover photo by Luís Paulo Rebelo.
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I declare that the work presented in this thesis, except where otherwise stated, is based on my own research. The work was mainly performed in the Applied and Environmental Mycology and Molecular Thermodynamics Laboratories of the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, between March 2009 and June 2013 and supervised by Doctor Cristina Silva Pereira (ITQB-UNL) and Professor Luís Paulo Rebelo (ITQB-UNL). Part of the results was attained during visiting periods to the Solution Chemistry Group of the Institute of Physical and Theoretical Chemistry, University of Regensburg, Germany and to the Macromolecular and Lignocellulosic Materials Group, Department of Chemistry, University of Aveiro.

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À minha Família,

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When I embraced this project, I was far from imagining how hard and gratifying science could be. Now, looking back for the past four years I am glad to realise that, despite rather ambitious, I was able to achieve the goals I committed with. It was four years of striving, patience and persistence. I must also recognise that these achievements wouldn't have been possible without the endless support of some people, some of which were and will certainly be an inspiring source of motivation.

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I would like to express my gratitude towards Fundação para a Ciência e Tecnologia for providing the financial support and Instituto de Tecnologia Química e Biológica, my host institution, for providing the facilities, services and resources to make this work possible. The people I met in this Institution throughout these years have definitively contributed (directly or indirectly) to create the excellent conditions and atmosphere for doing research. My thanks and appreciations also go to all former and present members of the Applied and Environmental Mycology Laboratory and of the Molecular Thermodynamics Laboratory. I would also like to leave a special word of encouragement to my colleagues who have been involved and will continue the development of this project. To "beer hours" and football (lab)mates, thanks for providing such good moments. I'll miss you.

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Table of Contents

Creating greater value for biomass residues

Extraction of suberin and betulin using alternative solvents

Abstract: Creating greater value for biomass residues – extraction of suberin and betulin using alternative solvents

Renewable resources have been the focus of both the scientific and industrial communities as substitutes for fossil fuels. Numerous studies have addressed the conversion of biomass to value-added chemicals, to materials, to fuels and into power and/or heat, *i.e.* biorefinery. The main goal of this thesis is to develop alternative extraction processes to isolate value-added constituents from biomass residues. Cork and birch outer bark were selected since the corresponding processing industries generate significant amounts of residues with high contents of value-added compounds, namely suberin (structural component) and triterpenes (extractable components).

One of the most challenging problems when developing extraction processes is the choice of a solvent that is simultaneously efficient and selective. Due to their unique properties, ionic liquids offer novel potential. This heterogeneous group of chemicals, composed solely of ions, can be designed to address the particular requirements of the process *e.g.* polarity, solvation ability and selectivity for a specific solute. In fact, the rational design of ionic liquids has allowed them to succeed in processes where common solvents and catalysts failed or displayed only limited performance.

Suberin, a complex aromatic-aliphatic cross-linked biopolyester, accounts for about half of the dry weight of cork and birch outer bark. This biopolymer has remarkable potential due to specific characteristics including its composition, its hydrophobicity and its high thermal resistance. In this thesis, it was demonstrated, for the first time, that some ionic liquids can selectively and efficiently extract suberin from cork and birch outer bark (Chapter II, III and IV). Cholinium hexanoate was selected as the most adequate solvent since it demonstrated both excellent suberin extraction efficiency and high bio-compatibility/degradability. In addition, preliminary data showed that this ionic liquid could be recovered and reused, further assuring the sustainability of the process. Extensive characterisation of the recovered suberin after its extraction by cholinium hexanoate revealed that it displayed distinct structural features when compared to that isolated by conventional processes (Chapter III and IV). A clear demonstration that

suberin depolymerisation by the ionic liquid progressed through ester bond partial hydrolysis was herein attained, both experimentally and theoretically (*ab initio* calculations) (Chapter IV). The data showed that the most favourable reaction is the hydrolysis of acylglycerol ester bonds, whilst most of the other aliphatic ester bonds remained intact. This explains the partially preserved structure of suberin extracted by the ionic liquid, as opposed to the highly depolymerised suberin obtained by conventional methods. Notably, suberin isolated by this alternative process displayed, for the first time, film-forming ability (Chapter V). The study emphasises the dual roles of the ionic liquid during suberin depolymerisation, both as a solvent and as a catalyst.

Finally, an alternative process for the isolation of triterpenes, in particular betulin, was also developed. Betulin is particularly abundant in the outer bark of birch species and exhibits a wide spectrum of biological activities such as antitumor and anti-HIV properties. Betulin was efficiently and selectively isolated from birch outer bark through microwave assisted extraction using limonene as solvent (Chapter VI). This method promises savings in time, energy and solvent when compared to the traditional Soxhlet extractions.

The integration of both alternative methods presented here, extraction of triterpenes and of suberin, may greatly stimulate the perceived value of both studied residues. These processes illustrate that natural products can be competitively extracted through the use of alternative solvents/techniques. While they represent remarkable scientific breakthroughs, the suberin depolymerisation in an ionic liquid was the critical groundwork for producing suberin-based materials. Notably, the knowledge obtained should be successfully applied to the extraction of other triterpenes (*e.g.* friedelin) and biopolyesters (*e.g.* cutin) from numerous biomass residues. The selective hydrolysis of ester bonds reported may also foster the development of new catalytic processes in ionic liquid media.

Resumo: Valorização de resíduos de biomassa - extração de suberina e betulina utilizando solventes alternativos

Os recursos renováveis têm estado no foco da comunidade científica e industrial como substituintes dos combustíveis fósseis. Inúmeros estudos têm abordado a conversão de biomassa em produtos químicos de valor acrescentado, materias, combustíveis, energia e/ou calor, *vide* biorefinaria. O principal objectivo desta tese é o desenvolvimento de processos de extracção alternativos para o isolamento de compostos de valor acrescentado a partir de resíduos de biomassa. A cortiça e a casca externa da bétula foram as fontes de biomassa selecionadas uma vez que as respectivas indústrias processadoras geram grandes quantidades de resíduos com elevado teor em compostos de valor acrescentado, nomeadamente suberina (elemento estrutural) e triterpenos (compostos extractáveis).

Um dos maiores desafios no desenvolvimento de processos de extracção é a selecção de um solvente que seja simultaneamente eficiente e selectivo. Os líquidos iónicos oferecem devido às suas propriedades únicas, novas potencialidades. Este grupo heterogéneo de compostos químicos, constituídos apenas por iões, pode ser desenhado para preencher os requisitos específicos de um processo, como, polaridade, capacidade de solvência e selectividade para um determinado soluto. De facto, a concepção racional de líquidos iónicos permitiu que estes fossem bem sucedidos em processos onde solventes orgânicos e catalisadores convencionais falharam ou apresentaram desempenhos limitados.

A suberina, um polímero aromático-alifático complexo e reticulado, representa cerca de metade da massa de cortiça e de casca externa de bétula. Este biopolímero tem um potencial notável devido às suas características, tais como composição química, hidrofobicidade e alta resistência térmica. Nesta tese, é demonstrado, pela primeira vez, que alguns líquidos iónicos conseguem extrair suberina de modo eficiente e selectivo a partir de cortiça e de casca externa de bétula (Capítulos II, III e IV). O hexanoato de colínium foi selecionado como o solvente mais adequado uma vez que apresenta excelente capacidade de extração de suberina e alta bio-compatibilidade / degradabilidade. Além disso, dados preliminares mostraram que este líquido iónico pode ser recuperado e reutilizado, reforçando a sustentabilidade do processo. A caracterização detalhada da suberina

recuperada após extracção com hexanoato de colínium revelou que esta exibe características estruturais distintas daquelas apresentadas pela suberina isolada por processos convencionais (Capítulo III e IV). É ainda demonstrado, tanto experimentalmente como teoricamente (cálculos *ab initio*), que a despolimerização da suberina pelo líquido iónico evolui através da hidrólise parcial de ligações éster (Capítulo IV). Os dados mostram que a reacção mais favorável é a hidrólise das ligações éster do tipo acilglicerol, enquanto a maioria das outras ligações éster alifáticas permanece intacta. Isto explica a estrutura parcialmente preservada da suberina quando extraída com este líquido iónico, em oposição à suberina altamente despolimerizada obtida por métodos convencionais. Notavelmente, a suberina isolada por este método alternativo exibe, pela primeira vez, capacidade de formar filmes (Capítulo V). O estudo realça ainda a dupla função desempenhada pelo líquido iónico durante a despolimerização da suberina, quer como solvente quer como catalisador.

Por fim, foi também desenvolvido um processo alternativo para o isolamento de triterpenos, nomeadamente de betulina. A betulina é particularmente abundante na casca externa de bétula e exibe um amplo espectro de actividades biológicas, tais como as propriedades antitumorais e anti-HIV. A betulina foi isolada de modo eficiente e selectivo da casca externa de bétula através de extração assistida por microondas usando limoneno como solvente (Capítulo VI). Quando comparado com a tradicional extracção Soxhlet, este método permite economizar tempo, energia e solventes.

A integração dos dois métodos alternativos aqui apresentados, ou seja, a extração de triterpenos e de suberina, pode aumentar consideravelmente o valor imputado aos resíduos estudados. Estes processos reflectem a possibilidade dos produtos naturais serem extraídos de forma competitiva utilizando solventes / técnicas alternativas. Ambos representam avanços científicos notáveis, não obstante a despolimerização de suberina num líquido iónico constitui a base fundamental para a produção de materiais feitos deste biopolímero. De realçar que o conhecimento adquirido pode ser aplicado com sucesso na extracção de outros triterpenos (por exemplo fridelina) e biopoliésteres (por exemplo cutina) de imensos resíduos de biomassa. A hidrólise selectiva de ligações éster apresentada nesta tese pode também promover o desenvolvimento de novos processos catalíticos em líquido iónico.

Thesis Outline

Chapter I – Introduction

The framework behind this thesis is presented and the concepts which fall within the scope of the following chapters are described: alternative methods for the extraction of chemicals from biomass residues.

Chapter II - Dissolution of cork biopolymers in biocompatible ionic liquids

Biocompatible and biodegradable cholinium alkanoates, in particular cholinium hexanoate, were demonstrated to efficiently and selectively extract suberin from cork.

Chapter III – Suberin isolation process using cholinium hexanoate

Suberin extraction either from cork or birch outer bark can be successfully attained with cholinium hexanoate. The data highlights the versatility of the process. Obtained suberin is partially depolymerised, exhibiting an esterified and cross-linked nature.

Chapter IV – Unveiling the dual role of cholinium hexanoate as solvent and catalyst in suberin depolymerisation

Cholinium hexanoate catalyses the hydrolysis of suberin ester bonds, in particular acylglycerols, explaining why upon extraction the cross-linked and esterified structure of suberin was partially preserved. Cholinium hexanoate acts simultaneously as a solvent and a catalyst.

Chapter V- Biomimetic suberin as novel hydrophobic antimicrobial materials

Ex-situ reconstitution of suberin as a material was attained. Suberin films are mimetic of the suberin barrier in plant cell walls, *i.e.* they are water-proof and show antimicrobial properties.

Chapter VI - Microwave assisted extraction of betulin from birch outer bark

High pure betulin raw extracts, *ca*. 95%, were isolated from birch outer bark through microwave assisted extraction with limonene.

Chapter VII - Discussion: Impact and Perspectives

A critical evaluation of the work described in this thesis is given, highlighting its scientific impact, possible improvements and the "greenness" of the developed processes.

Members of the Jury

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Publications

Thesis publications

R. Ferreira, H. Garcia, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. Nimal Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers by biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367 – 369.

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Additional publications

M. Petkovic, J. L. Ferguson, A. Bohn, J. R. Trindade, I. Martins, M. C. Leitão, M. B. Carvalho, C. Rodrigues, H. Garcia, **R. Ferreira**, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Exploring fungal activity in the presence of ionic liquids, *Green Chem.*, 2009, **11**, 889–894.

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C. Silva Pereira, **R. Ferreira**, H. Garcia and M. Petkovic, Ionic Liquids - Biocompatible, in Encyclopedia of Applied Electrochemistry, *Springer* (*available online: http://www.springerreference.com/docs/html/chapterdbid/303469.html*).

Chapter I

Introduction

Subsection 6 contains parts of the following publications of the author: R. Ferreira, M. Blesic, J. Trindade, I. Marrucho, J. N. C. Lopes and L. P. N. Rebelo; Solubility of fluorinated compounds in a range of ionic liquids. Cloud-point temperature dependence on composition and pressure; *Green Chem.*, 2008, 10, 918–928; R. Ferreira, H. Garcia, M. Petkovic and C. Silva Pereira; Ionic Liquids: Biocompatibility; Encyclopedia of Applied Electrochemistry, *Springer* (available online)

1. Objectives

The biorefinery and green chemistry constitute permanently evolving fields. Despite these topics have been continuously revised throughout the last decade, a comprehensive assessment and evaluation of the progresses is rather difficult. These are certainly evidences of the growing awareness that a change to a sustainable path needs to be made soon. Regardless of how far we are of the shift from petroleum hydrocarbons to bio-based feedstock, it is the responsibility of research to prove concepts and to break technological barriers. The present thesis aims to contribute to these evolving fields by focusing on the extraction of value-added chemicals from industrial plant residues. Namely, the extraction of **suberin** from **cork** and **birch outer bark** using **ionic liquids** as solvents (Chapter II to V) and the microwave assisted extraction of **betulin** from **birch outer bark** (Chapter VI). In the following subsections the concepts which fall within the scope of this thesis will be addressed in further detail.

2. The context: biorefineries and green chemistry

One-hundred-and-fifty years after the beginning of the fossil fuel realm, society is now facing a new paradigm shift. The growing demand for energy, fuels and chemicals has been largely met by fossil resources. However, it is unquestionable that at the current consumption rates these sources will be depleted within some generations[1,2]. This perception together with the nonexistence of a cost-efficient alternative to fossil sources, has given rise to geopolitical tensions, market speculation and to an overall increase of fuel prices. Furthermore, the detrimental effect on environment associated with the use of these non-renewable resources has awakened a sense of awareness regarding sustainability. Given this well-known state of affairs, a forward looking strategy is the progressive foundation of a sustainable economy supported on bioenergy, biofuels, and bio-based chemicals. Whereas for energy production there are a number of alternatives, *e.g.* solar, hydraulic, wind, geothermal and nuclear energies, the chemical and fuel industries rely on the conversion of renewable materials. In this scope, biomass has great promise and represents the only foreseeable source of feedstocks, since it is massively

renewed within a useful time scale, *i.e.* 170 gigatones renewed yearly from which only *ca.* 6 are used, mainly in the food sector.[3]

The exploitation of renewable resources, even at industrial levels, is not a new concept. Well before the petrochemical revolution, the progresses of chemistry had already led to a variety of processes based on natural sources (some still running without direct competition). These include, for instance, paper pulping, starch, sucrose and vegetable oils processing, the vulcanization of natural rubber and the production of cellulose ester thermoplastics.[4,5] With the upsurge of fossil resources, the chemical industry became swamped with easily accessible chemicals and fuels. As a result, synthetic materials with undeniable utility in modern life, such as plastics and packaging materials were produced at low-costs, displacing most bio-based products to a non-competitive level. Since then, modest economic (and intellectual) investment was devoted to the industrial use of natural resources, in opposition to the multi-billionaire sums invested in petrochemistry. Recently, with the mandatory (and somehow fashionable) resurgence of interest in renewable resources, industrial practices analogous to petroleum refineries have been proposed – the "biorefinery" concept was coined.[4] These facilities comprise the processes and equipment necessary for biomass conversion into fuels, power, heat, and materials, including food, feed, and chemicals. The underlying question at this point is how to explore plant biomass to produce bio-commodities and specialty chemicals in a cost competitive and sustainable way, *i.e.* without leading to ecological and/or social imbalances. With that aim, the so-called integrated (or phase III) biorefineries have been foreseen. These correspond to the most advanced and desirable scenario[4,6,7]. Thermochemical, biochemical, and chemical technologies are combined in a flexible process which allows full conversion of variable feedstocks into a spectrum of products and energy causing minimal ecological footprint.

Polysaccharides, namely cellulose, and lignin have been the primary focus of biorefineries[4-7] since they represent the most abundant natural polymers. Yet a representative list must also include hemicellulose, starch, chitin, chitosan, extractives

such as terpenes, proteins, polycarboxylic acids, vegetable oils and polyesters such as cutin, suberin, shellac or bacterial poly(hydroxyalkanoates). According to their structural features and end-use, these compounds can be modified, used without further treatments or converted into bulk and/or building-block chemicals[8,9], *e.g.* 5-hydroxymethylfurfural, γ-valerolactone, furfural, glycerol, sorbitol, levulinic acid, succinic acid and lactic acid.[5,10-12] Some bio-based products, such as, biodiesel, ethanol, poly(lactic acid) and 1,3-propanediol, are already cost-competitive when compared to the petroleum-based equivalents.[7,10,13] However, their impact is limited within the still running petroleum-based technologies. Thus, many countries have committed with regulatory practices[14-16] and with short- and medium-term binding targets within policy programs[1,6]. These efforts have been accompanied by private and public investment which has resulted in an exponential increase of scientific output. Nevertheless, despite these progresses, through a realistic analysis one can easily recognise that we are still in the early stage of the transition from an oil-based economy to a bio-based economy.

The change to a sustainable path requires the rearrangement of entire processes and completely new approaches. New synergies between agriculture, plant sciences, genetics, green chemistry, white biotechnology and process engineering need to be established and will require great endeavour and engagement. Yet, the necessary scientific knowledge and technology seems achievable. In the long term, intense and sustainable exploitation of renewable resources will possibly involve genetically modified plants. These will be tailored to combine high biomass production yields with fast growth and resistance to biotic and abiotic stresses such as drought, cold or pathogenic attacks[13,17]. Enzymatic and chemical deconstruction steps will be determinant to overcome the recalcitrance of plant biomass and to its conversion into building-block chemicals. As a consequence of the complexity of the resulting mixtures, the development of new purification processes is envisaged[18]. In fact, the downstream processing constitutes one of the biggest obstacles in biorefineries and is estimated to account for 60 to 80 % of the costs.[17] While in petroleum refineries, distillation is the main unit operation, in biorefineries the non-volatility of most biomass components

hampers the direct use of this simple separation procedure. Thus, the integration of techniques such as solvent extraction, membrane filtration, adsorption and chromatography will certainly play here a key role.[19]

Closely related to the concept of biorefinery is the concept of green chemistry. This encompasses the "*design of chemical processes and products to reduce or eliminate the use or generation of hazardous substances. Green chemistry applies across the life cycle of a chemical product, including its design, manufacture, and use*".[20] Accordingly, Anastas and Warner proposed guidelines for the design of chemical synthesis and new compounds. The core ideas are embodied in the "12 Principles of Green Chemistry"[21]: (*1*) prevention, (*2*) atom economy, (*3*) less hazardous chemical synthesis, (*4*) design safer chemicals, (*5*) safer solvents and auxiliaries, (*6*) design for energy efficiency, (*7*) use of renewable feedstocks, (*8*) reduce derivatives, (*9*) catalysis, (*10*) design for degradation, (*11*) real-time analysis for pollution prevention, and (*12*) inherently safer chemistry for accident prevention.

Designing safer solvents (5th Principle of Green Chemistry), is probably one of the most active areas of green chemistry research.[21-23] Solvents constitute a significant fraction of processes wastes and their recyclability often requires energy consuming operations. Their volatility, toxicity and/or flammability have posed significant health and safety issues, namely when accidental spillages occur. Moreover, such properties have also contributed significantly to air, water and soil pollution. Aiming to improve the sustainability of chemical processes, solventless systems, water, supercritical fluids and ionic liquids have been proposed as alternatives to common organic solvents.[21,22] For obvious reasons, the first option is the most desirable approach, although it constitutes an extreme technological challenge that requires a complete redesigned chemistry.[21,24,25] The use of water[26] and supercritical fluids[27,28] has been intensively investigated and despite some limitations inherent to their properties, successful results have been obtained.[23] Similarly, ionic liquids have also shown great promise.[29,30] These solvents display unique properties, when compared to any of the previous options. They exhibit negligible vapour pressure at and near room-temperature[31], which *a priori* eliminates any leakage through

evaporation, and can be designed to be simultaneously efficient towards a specific process and benign – Principles of Green Chemistry 4, 5 and 10.

In order to evaluate the environmental acceptability of chemical processes simple metrics were proposed as directing tools. Sheldon introduced the E-factor, defined as the mass ratio of waste to desired products,[22,32] while Trost introduced the atom economy, defined as the ratio between the molecular weight of the desired products and the sum of the molecular weights of all products[33]. As aforementioned, these are simple guiding tools, therefore a detailed evaluation should consider the whole process, from efficiency and safety of chemical reactions, to legislation and life cycle assessment of raw materials and involved chemicals.[21,34] Emphasis should be also given to the fact that the use of renewable resources does not fulfil the Principles of Green Chemistry *per se*. Instead, the full process should be accounted for. Some large companies such as BASF and GlaxoShmithKline have moved on this direction and developed assessment tools for solvent selection[35] and for monitoring environmental, economic and social impact of the biomass processing[36], *viz.* "Ecoefficiency analysis" and "Seebalance" analyses[37].

3. *Quercus suber* L. and *Betula pendula* R. as sources of biomass residues

In this thesis, the potential of *Quercus suber* L. and *Betula pendula* R. outer barks as sources of useful chemicals was investigated. The following lines describe characteristics of these two tree species and highlight the amounts and composition of biomass residues generated in their processing industries.

3.1 Birch outer bark

Betula pendula Roth, commonly known as silver birch, is a deciduous tree species from the *Betulaceae* family. It occurs naturally in Europe and Asia but is particularly abundant in the temperate and boreal forests of Northern European countries, where it accounts for up to 28 % of the total volume of the growing stock. Silver birch can be grown either in mixed or pure stands and presents a fast early growth, reaching a height of 20-30 meters between 30 and 50 years. After this period, growth starts to decline.[38]

Silver birch is widely used in paper pulping industries in the Baltic and Nordic countries for timber and plywood production, constituting the most important tree species for commercial practice in this region. Prior to processing, bark is removed from the logs. Currently, this fraction is used simply as a cheap fuel to produce energy in biomass boilers.[39] However, substantial valorisation can be attained if valuable components are extracted prior to burning. In mature birch trees, bark accounts for about 15 % of the logs' weight, including 3.4 to 5.4 wt% of outer bark and 6.1 to 12 wt% of inner bark.[40,41] Along with lignin (10-30 wt%) and polysaccharides (10-30 wt%), birch outer bark comprises two value-added fractions, namely suberin (30-50 wt%), and extractives (20-40 wt%).[40-45] The isolation of these products would however require the separation of the enriched outer bark from the inner fraction. This can be achieved manually or gravimetrically. While the "hydrophobic" outer bark floats on the surface of water, the inner bark sinks.[41] Separation can also be attained by bark milling followed by sifting of the outer fraction, which is more resistant to grinding when compared to the inner bark.[39]

Based on the current figures, birch outer bark can be regarded as a virtually unlimited source of extractives and suberin monomers. For instance, from a mill that processes 400 000 tonnes of birch per year, it would be possible to obtain *ca.* 20 000 tonnes of outer bark, corresponding to *ca.* 8000 tonnes of suberin and 6000 tonnes of extractives.[39,41]

3.2 Cork

Quercus suber L., known as cork oak, is a slow-growing evergreen tree species native to western Mediterranean area. It is however predominantly present in the south region of Portugal[46], where it accounts for about one third of the world's cork oak forest area (*ca.* 700 000 ha)[47,48]. *Quercus suber* L. can be grown either in mixed or pure stands achieving 14-16 meters height and a longevity of 250-350 years. Yet, the age of 150 - 200 years is regarded as the limit for industrial practice. This species is characterised by the presence of a rough bark that can grow up to several centimetres thick. The outer part of this layer, the phellem, is known as cork and constitutes a natural barrier that protects the plant from the surrounding environment.[49] Cork is arranged in an

alveolar structure of contiguous hollow cells characterised by the presence of suberised thick secondary walls. Despite some biological variability attributed to factors such as tree age, climate and soil conditions, suberin is found as the major constituent of cork, accounting for *ca.* 40 wt%. Lignin, polysaccharides and extractables account for *ca.* 20, 20 and 15 wt% respectively.[42,46,50-52]

Cork is periodically harvested from the tree, first stripping occurs approximately at the age of 25 years and then every 9 to 15 years. Upon removal, the formation of a traumatic phellogen occurs leading to the formation of a new periderm, and therefore of a new cork layer.[46] This singular characteristic of *Quercus suber* L., together with the remarkable properties of cork, such as elasticity, low density and significant microbial resistance, constitutes the basis for its commercial exploitation as a raw material. First and second cork stripping yield low quality cork, *i.e.* virgin and second cork respectively, while the subsequent cork stripping produce a good quality material, known as reproduction or *amadia* cork).[46]

In fact, the cork sector represents one of the most dynamic economic activities in Portugal. Forest inventories indicate a world cork productions of *ca.* 2 hundred thousand tonnes per year from which more than half is attributed to Portugal, representing more the 800 M€ in exports, 130 M€ in imports and a trade balance of 670M€.[47]

Cork is mainly used for the production of stoppers (40 wt%) and other cork-based products (35 wt%) including agglomerates, flooring and insulation panels, coverings, cubes, plates, sheets and strips.[47] During cork processing, large amounts of residues (25 wt%) are generated,[47] namely industrial cork powder and black condensate. The first is chemically similar to natural cork and comprises the material that does not have a proper size for agglomerate production or further uses.[53-55] The second represents the volatile compounds released during the production of black agglomerates. During this process, cork granules are extensively heated (above 250 ºC) causing its adhesion, and the simultaneous formation of vapours, which later condense in autoclave pipes forming the black condensate. This fraction requires periodic removal[54] and is enriched in biologically active triterpenes[55,56]. Overall cork powder and black condensate account respectively for *ca.* 40 000 and 2500 ton/year in Portugal. These residues are usually burned for energy

production.[53,54] However, when considering their composition and the amounts of residues generated, one can easily recognise their potential use as sources of value-added chemicals.[55]

Both cork and birch outer bark share the distinctive feature of being suberised tissues. The cells of these materials comprise lignocellulosic primary walls, suberised secondary walls and cellulosic tertiary walls. In addition, an essentially lignified structure, the middle lamella, keeps the cells together. The secondary walls account for most of the cell wall thickness and have a preponderant role in the physiological function of these plant-environment interfacial tissues.[46,57,58] Low molecular weight non-structural elements, the extractives, are deposited either in the inner face of the cell walls or associated with suberin in the secondary wall.[57] As aforementioned, suberin and the extractive fraction of these residues are regarded as promising value-added products. Therefore, in the following subsections these two fractions will be described in further detail.

4. Suberin

Note: Two sets of polymeric structures are found in suberised secondary cell walls, one polyaromatic and one polyaliphatic (with associated phenolics). Debate still persists about whether the term suberin should include the two sets of polymeric structures or only the polyaliphatic. Since the presence of the polyaromatic domain is a distinct feature of suberised tissues and since it is chemically different from the other cell wall biopolymers, namely lignin, the term suberin is herein used to describe the whole macromolecular structure - polyaromatic and polyaliphatic domains and the phenolics associated with the latter. It is however recognised that common depolymerisation methods rely on the ester-bonds cleavage and consequently only lead to the extraction of the polyaliphatic domain. Therefore in the scope of the extraction of this biopolymer from the plant cell walls, the suberin term is used to refer to the isolated material, regardless of its nature (aliphatic and/or aromatic) and of the extraction yields.

Suberin is a ubiquitous biopolyester found in higher plants, and particularly abundant in the outer barks of *Quercus suber* and *Betula pendula* and in the peels of *Solanum tuberosum.* It constitutes the major structural element of these plant-environment interface tissues accounting for up to *ca.* 50 % of their weight.[41,42,52,59,60] This lipophilic biopolymer is deposited in the internal and peripheral dermal tissues during secondary cell wall differentiation or as a response to stress and/or wounding.[46] Moreover the importance and complexity of such structure is deeply related to its physiological role in plants where it constitutes an apoplastic barrier that controls the flow of water, gases and solutes and protects the plant against microbial attack and physical aggressions.[49,58,61-64]

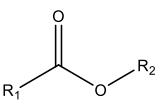
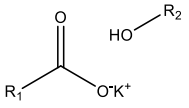
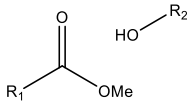
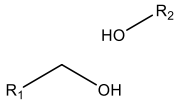
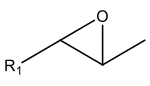
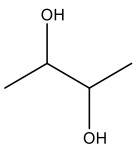
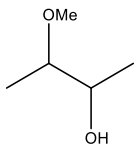
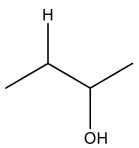
Suberin can be described as a naturally occurring network comprising two spatially segregated domains extensively linked between each other and to the other cell wall components.[52,57,58,65-67] One shows a polyaliphatic nature and is essentially a glycerol-based polyester of ω-hydroxyacids and α,ω-dicarboxylic acids.[68] The other, polyaromatic, constitutes a recalcitrant lignin-like network.[66,69] Yet, the promise of this lipophilic biopolymer as a source of chemicals has been mostly related to the polyaliphatic domain, namely to the high abundance of polyfunctional monomers. These are appealing structures for polymer synthesis, though rare in nature and difficult to prepare chemically. *In situ* suberin is however an insoluble biopolymer highly resistant to chemical or enzymatic treatments and difficult to isolate as a pristine material. Hence, with the aim of extracting and characterising suberin, several techniques have been developed to isolate it from the cell walls.

4.1 Depolymerisation Methods

The recalcitrance of suberin and its extensive linkages to the other cell wall components makes depolymerisation an essential step either for its extraction from plant tissues or for its detailed chemical characterisation. In accordance with its polyester nature, suberin depolymerisation is based on simple ester bond cleavage reactions, namely hydrolysis (saponification), alcoholysis (transesterification) or reductive cleavage (Table 1).[52,64] These induce different chemical modifications on suberin monomers and

may result in variable extraction yields. Therefore, the selection of the depolymerisation method and conditions greatly depends on the purpose of the study: extract or quantify the whole suberin present in the plant matrix, structural elucidation studies or monomeric analysis. Generally, there is a balance between suberin extraction yield and the degree of depolymerisation. When suberin full removal is desired, methods that lead to complete depolymerisation are generally favoured. These usually involve harsh chemical conditions, and thereof extensive ester bond cleavage, yielding suberin mainly in the form of monomers.[52] Instead, for structural elucidation studies partial depolymerisation methods are preferred.[68,70] These can selectively cleave specific moieties[71,72] or involve milder conditions, releasing dimeric and/or oligomeric suberin structures[68,70]. Although out of the scope of this thesis, one should not disregard that suberin enriched samples can also be obtained by digesting the polysaccharides from the cell wall, namely with cellulases, hemicellulases and pectinases. Despite most frequently applied to the isolation of suberin from potato periderms, this procedure has also been applied to cork suberin.[73] Although obtained in a polymerised form, the isolated fraction represents only *ca.* 10 wt% of the whole cork suberin.

Table 1| Products resulting from suberin depolymerisation reactions.

		Alkaline Hydrolysis†	Alcoholysis†	Hydrogenolysis
Ester group				
Epoxy ring‡				

† Under specific conditions, partial depolymerisation may occur and/or the epoxy rings can be virtually preserved.
‡ The epoxy ring opening results in random functionalization of the vicinal carbons.

Despite the chemical and biological variability of suberin sources, one cannot disregard factors such as grain size or depolymerisation conditions since these certainly have considerable effect in the overall reaction kinetics. Also noteworthy is the presence of considerable amounts of (non-covalently bonded) extractives in suberised tissues. Therefore, suberin depolymerisation is usually preceded by the removal of these soluble components, usually through Soxhlet extraction. The pre-treated product is an essentially insoluble matrix of polysaccharides, lignin and suberin.

Methanolysis

Alkaline methanolysis catalysed by sodium methoxide is by far the most favoured suberin depolymerisation method (Table 1). This reaction consists of a transesterification, therefore suberin is extracted in the form of methyl esters. When using *ca.* 0.05 to 3 wt% sodium methoxide refluxing in methanol from 2 to 3 hours, extensive ester bond depolymerisation occurs allowing complete removal of suberin.[45,71,74] Instead, lower concentrations of sodium methoxide have been shown to result in limited extraction yields and in the preferential release of alkanoic and α,ω-alkanedioic acids.[71,72] Mild depolymerisation conditions can also be attained when using calcium oxide or calcium hydroxide as catalysts.[68,70,75-78] These processes result in partial depolymerisation and despite the lower extraction yields, typically below 10 % of the initial bark weight, considerable amounts of dimers and trimers are released.[68,70,75,77-79] Such structures are of significant importance since they have given crucial insights into the structural organisation of *in situ* suberin.

Depending on the used methanolysis conditions, epoxy rings may be preserved or readily converted to their corresponding methoxyhydrins, a derivative that is absent in native suberin.[45] Thus, epoxy containing compounds may be unambiguously identified since they can only be detected as such or as the corresponding methoxyhydrins derivatives. This observation constituted a significant breakthrough when compared to the results obtained with alkaline hydrolysis (where the opening of the epoxy ring resulted in the corresponding *vic*-diol units, likely to be present in native suberin – see below, subsection Hydrolysis). For the abovementioned reasons, alkaline methanolysis has been

considered the reference method when considering identification and quantification of suberin structures.

Other alkaline alcoholysis methods have been reported. Depolymerisation can be readily achieved with sodium hydroxide[80,81] or potassium hydroxide[81,82]. Under these conditions, conversion of epoxy ring into methoxyhydrin occurs however the reaction was reported to be highly sensitive to moisture[81]. Similar results can be achieved through acid methanolysis with sulphuric acid[81], hydrochloric acid[83] or boron trifluoride[84,85] however, in acidic media the reaction rates are reported to be slower when compared to alkaline hydrolysis[81].

Hydrolysis

Complete depolymerisation and suberin removal can also be obtained by alkaline hydrolysis either in water or in aqueous alcoholic solutions. These processes consist in the saponification of the monomers, followed by acidification and extraction of the ensuing acids with organic solvents. Most commonly, hydrolysis is carried out in 3 wt% ethanolic potassium hydroxide under solvent reflux (Table 1).[41,42,45,60,81,86] Holloway showed that in these conditions the unstable epoxy-containing monomers may undergo acid- or base-catalysed conversion into the corresponding ethoxyhydrins and/or *vic*-diols.[45] Owing to the native occurrence of such *vic*-diol monomers in suberin, alkaline hydrolysis is often considered unsuitable for quantitative assessment of suberin composition.[45] Moreover, the use of strong mineral acids in the acidification step may result in additional artefacts. For instance, when hydrochloric acid is used it is likely to occur the formation of halohydrins whereas the use of sulphuric acid also resulted in the acid-catalysed conversion of epoxy into *vic*-diol.[45] The extent of epoxy ring cleavage depends on the nature and concentration of the catalyst and of the mineral acid used for the acidification as well as on the duration and temperature of the hydrolysis. It was however demonstrated by Ekman that these structures can be virtually preserved under specific circumstances. Namely, hydrolysis with 0.5 M potassium hydroxide in ethanol : water (9:1) followed by acidification with diluted sulphuric acid.[41,81] Under these conditions, suberin can be rapidly and completely depolymerised yielding

chemically unmodified monomers.[81] When the depolymerisation is performed in equivalent aqueous alkali conditions, hydrolysis progresses with a slower kinetics, considerable fraction of the epoxy rings are hydrolysed to *vic*-diols[81] and large amounts of dark coloured polyphenols are released[60,81]. As described by Coquet *et al.*, suberin hydrolysis can also be catalysed in acidic media, however under the used harsh conditions severe degradation of monomers may occur.[83]

Other depolymerisation methods

Suberin depolymerisation can also be attained through hydrogenolysis with lithium aluminium hydride in tetrahydrofuran. This reaction is highly destructive since it reduces the esters and epoxy moieties to alcohols as depicted in Table 1.[64,84,85,87] Therefore, structural information is lost during this process, for instance α,ω-alkanedioic acids and ω-hydroxyacids are reduced to similar diols. Yet reductive cleavage played a significant role for structural elucidation purposes. For instance, hydrogenolysis with deuterated lithium aluminium hydride introduces a deuterium in all reducible groups allowing the identification of their position.[85,87]

The aforementioned depolymerisation reactions lead to the cleavage of ester linkages. Accordingly, only the aliphatic monomers and ester-linked phenolics are released, while the stable ether and C–C (cross-)linked polyaromatic monomers remain attached to the cell wall. Therefore, harsh degradation methods typically applied in lignin were adapted to characterise the polyaromatic domain[88], *e.g.* thioacidolysis[66,69,89] or nitrobenzene oxidations[87].

4.2 Suberin monomeric composition

Suberin monomeric composition has been well described in numerous studies and relies mostly on its complete depolymerisation through conventional methanolysis or hydrolysis, followed by gas chromatography-mass spectrometry profiling. Alternatively, similar results can be attained by flash pyrolysis - gas chromatography - mass spectrometry in the presence of tetramethylammonium

hydroxide, a versatile and straight-forward technique that results in the hydrolysis of esters together with methylation of carboxylic and hydroxyl groups.[72,90] In general, results demonstrate some natural variability (even within the same species) and strongly depend on the used depolymerisation conditions and analytical techniques.[46,52,83] Moreover, as stressed by some authors,[46,71,74] in order to obtain accurate chromatographic quantification data, internal standards must be used and the response factors of the various suberin monomers should be accounted for. This procedure has been often ignored, and may also justify to some extent the observed compositional differences. Yet the relative abundance of monomers is comparable across all studies and shows a common pattern. Table 2 summarises representative data concerning monomeric composition of cork and birch outer bark suberin, as detected from hydrolysis and methanolysis depolymerisations. These procedures yield an aliphatic mixture of even numbered C_{16} to C_{26} chains essentially composed of ω-hydroxyacids, α,ω-alkanedioic followed by minor amounts of alkanoic acids and alkanols (Figure 1, Table 2). Among these, the C_{18} ω-hydroxyacids and α,ω-alkanedioic acids bearing *mid*-chain unsaturated, epoxy or *vic*-diol derivatives are the most abundant, followed by the C_{22} homologues (Table 2). Glycerol is also present in significant amounts, from 2 to 14 % of suberin weight.[68,74] However, its presence is often ignored or overlooked by monomeric quantification studies since this polyol is unintentionally discarded during common suberin isolation protocols. Despite being long recognized as a suberin monomer, the importance of glycerol as a key cross-linker in the formation of suberin three-dimensional structure was only identified in the end of the last century by Graça and Pereira.[68] In fact its role is fully emphasised by the observation that, when compositions are expressed in molar ratio, glycerol constitutes the most abundant suberin monomer.[46] Even though detected in minor amounts, ferulic acid constitutes the most frequently found aromatic monomer.[52] One should bear in mind that since these depolymerisation techniques result in ester bond cleavage, only the ester linked monomers are depolymerised. Therefore, the recalcitrant polyaromatic domain monomers are most likely absent from these extracts.

Table 2| Relative abundance of aliphatic suberin monomers from the extractive-free cork and birch outer bark (adapted from Gandini *et al.*[52]). The notation "0" means that the compound was detected in trace amounts. References are indicated as follows: A[71], B[74], C[72], D[91], E[92], F[93], G[90], H[80], I[41], J[81], K[42].

		Cork								*Birch outer bark*		
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
Aliphatic alcohols		**4.7**	**0.4**	**2.2**	**4.5**	**8.3**	**1.0**	**1.8**	**1.8**	**0**	**0**	**0**
C(20:0)	Eicosanol	0.5	0.1	—	0.3	0.5	—	0.1	—	—	—	—
C(22:0)	Docosanol	2.0	0.2	1.7	3.2	3.1	—	0.7	0.9	—	—	—
C(24:0)	Tetracosanol	1.0	0.1	0.5	0.5	4.3	—	0.9	0.9	—	—	—
C(26:0)	Hexacosanol	0.5	—	—	0.5	0.4	—	0.2	—	—	—	—
Fatty acids		**14.9**	**1.0**	**2.5**	**4.9**	**14.1**	**8.0**	**4.2**	**4.7**	**12.3**	**7.4**	**0**
C(16:0)	Hexadecanoic acid	0.2	0	—	0.5	—	—	0.1	—	—	—	—
di(OH)-C(18:0)	9,10-dihydroxyoctadecanoic acid	1.3	—	—	—	6.6	2.0	—	—	—	—	—
C(22:0)	Docosanoic acid	1.3	0.9	2.0	1.7	1.5	—	2.5	1.8	—	—	—
C(24:0)	Tetracosanoic acid	1.1	—	0.5	0.5	0.4	—	1.5	1.4	—	—	—
ω-hydroxyacids		**51.5**	**26.3**	**36.0**	**44.0**	**46.5**	**58.0**	**61.7**	**40.8**	**76.7**	**79.7**	**86.3**
C(16:0)	16-hydroxyhexadecanoic acid	1.1	0.4	0	0.6	0.8	—	1.2	0.5	—	—	0.4
C(18:1)	18-hydroxyoctadec-9-enoic acid	8.8	5.4	11.1	9.7	10.3	—	18.2	5.7	11.1	12.2	15.3
9,10-epoxy-C(18:0)	9,10-epoxy-18-hydroxyoctadecanoic acid	4.6	7.3	5.5	2.1	1.8	5.0	3.2	7.5	37.0	39.2	38.8
9,10-di(OH)-C(18:0)	9,10,18-trihydroxyoctadecanoic acid	12.7	2.2	—	10.0	10.6	3.0	4.8	7.0	8.6	8.4	11.4
C(20:0)	20-hydroxyeicosanoic acid	2.2	0.5	0.7	0.9	0.9	—	0	1.2	2.8	2.8	3.5
C(22:0)	22-hydroxydocosanoic acid	16.3	7.9	17.4	11.7	13.4	—	28.6	15.4	13.6	13.9	16.5
C(24:0)	24-hydroxytetracosanoic acid	4.6	2.4	1.3	3.1	3.2	—	5.8	2.9	—	—	0.4
C(26:0)	26-hydroxyhexacosanoic acid	—	0.1	—	4.4	5.2	—	0	—	—	—	—
α,ω-dicarboxylic acids		**27.6**	**45.5**	**53.3**	**18.6**	**6.1**	**22.0**	**21.3**	**48.8**	**10.4**	**12.9**	**13.7**
C(16:0)	hexadecanedioic acid	1.6	2.0	2.2	—	0.5	—	3.1	2.2	—	—	0.4
C(18:0)	octadecanedioic acid	0.5	0.5	—	—	—	—	—	0.5	0.9	—	0.4
C(18:1)	octadecane-9-enedioic acid	4.1	6.2	7.7	2.1	—	—	9.1	7.1	3.4	4.7	5.1
9,10-epoxy-(C18:0)	9,10-epoxyoctadecanedioic acid	5.3	22.9	37.8	3.1	—	4.0	0.9	—	—	—	—
9,10-di(OH)-C(18:0)	9,10-dihydroxyoctadecanedioic acid	5.0	7.7	2.5	6.8	—	0	1.3	7.2	—	—	0
C(20:0)	eicosanedioic acid	2.6	1.0	1.1	4.9	3.8	—	1.2	1.5	—	—	1.6
C(20:1)	eicosane-9-enedioic acid	0.3	—	—	—	—	—	—	—	—	—	—
C(22:0)	docosanedioic acid	7.1	4.5	1.7	1.5	1.5	—	5.6	10.3	6.1	8.2	6.3
C(24:0)	tetracosanedioic acid	1.1	0.7	0.3	0.3	0.3	—	0.5	—	—	—	—
Aromatics - Ferulic acid		**1.3**	**0.5**	**3.9**	**6.6**	**7.9**	**6.0**	**0.1**	**0.8**	**—**	**—**	

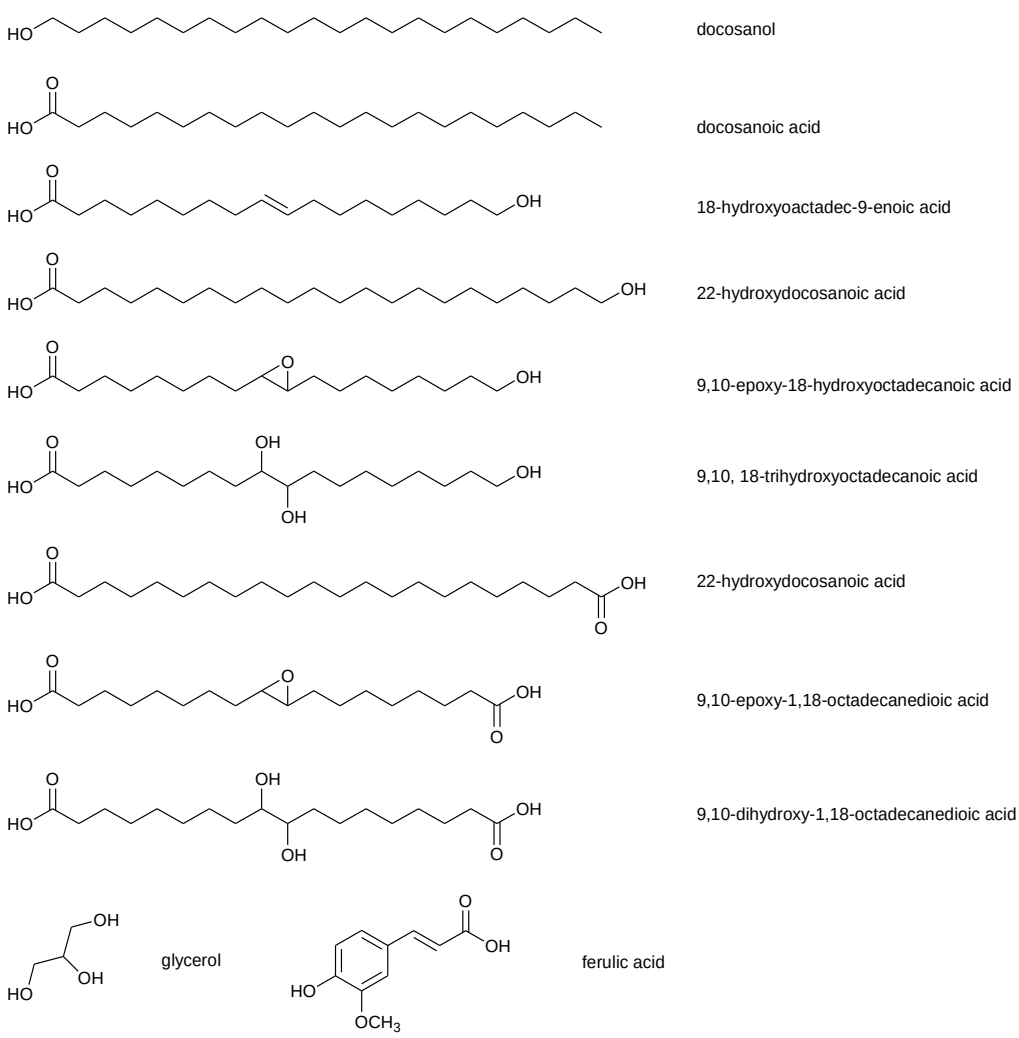


Figure 1| Representative aliphatic monomers of suberin tissues.

As aforementioned, gas chromatography - mass spectrometry has been selected as the reference analytical method for suberin monomeric identification. However, the amount of gas chromatography - mass spectrometry detectable monomers in respect to the total mass of depolymerised suberin is quite variable and is most often below 50 wt%.[42,71,74,80] This observation has been associated with the presence of non-volatile oligomeric structures, namely suberan, an insoluble and non-hydrolysable aliphatic macromolecular structure, suggested to present a high degree of crystallinity.[52,80,86,94]

Moreover, gel permeation chromatography and mass spectrometry studies revealed the presence of oligomeric structures with molecular weights high enough to hamper their detection by gas chromatography - mass spectrometry analysis.[72,80,46] In summary, a considerable fraction of suberin is not detected by this technique meaning that the chemical identity of the whole suberin sample is yet unresolved.

4.3 Suberin macromolecular assembly

The secondary wall constitutes essentially a thick suberised layer that comprises two spatially segregated domains extensively linked between each other and to the other cell wall components.[52,57,58,65-67] The first shows a lignin-like polyaromatic nature and is embedded in the inner face of the primary cell wall.[65,66] Due to its high recalcitrance, its composition and structure are not yet fully understood, even though, it is known to be composed mainly of hydroxycinnamic acids (predominantly ferulic acid) and their derivatives and by vestigial amounts of monolignols namely *p*-coumaryl, coniferyl, and sinapyl alcohols.[65,66,95] Monomers are extensively cross-linked between each other and to other cell wall components *via* stable C-C and amide linkages, and also through robust ether bonds as in lignin.[66,69,89]

Internally to the polyaromatic domain, sits an essentially aliphatic domain that contributes to most of the secondary cell wall thickness. The polyaliphatic domain is composed mainly of cross-linked units of ω-hydroxyalkanoic acids and α,ω-alkanedioic acids (and the corresponding *mid*-chain unsaturated, epoxy or *vic*-diol derivatives), glycerol, alkanoic acids and minor amounts of alkanols. Monomers are in a parallel alignment and linked *via* linear aliphatic ester or acylglycerol ester bonds, creating a hydrophobic environment.[52,57] Additionally, hydroxycinnamates esterified to glycerol or long chain ω-hydroxyacids are incorporated within this domain.[75,77-79,96] Importantly, the high abundance of polyfunctional monomers with terminal carboxylic and hydroxyl groups together with the presence of glycerol and *mid*-chain functionalities allows the structure to expand in a covalently linked three-dimensional network.[68,77,78] Microscope evidences revealed a lamellar ultrastructure within this domain[97] (despite not always

observed[98]) which has been associated to the presence of alternate layers of esterified aliphatics and aromatic structures.[58]

Although suberin monomeric composition has been known for many years, knowledge on its macromolecular assembly is still under debate and is considerably hampered by its highly cross-linked and heterogeneous nature. Most of the knowledge on this issue arises from the information collected by destructive protocols. In this context, the detection of dimers and/or trimers of linear esters, glyceryl esters and feruloyl esters (released by partial depolymerisation techniques) has been of utmost importance since they constitute experimental evidence of suberin monomeric assembly (Figure 2).[68,70,75-79] Even so, and notwithstanding the indisputable importance of these findings, these very small structures are not representative of the suberin structure as a whole. The analysis of the whole polymer through spectroscopic techniques, namely infrared[99] and nuclear magnetic resonance[65,67,100] spectroscopy, has contributed with important information. The latter gave insightful information regarding the presence and the nature of the two aromatic populations within suberin (the polyaromatic domain and the phenolics associated to the polyaliphatics)[52,66,67] and regarding the linkages between both suberin domains and to other cell wall components[65,67,71]. In fact these are still poorly understood, however it is generally accepted to involve either ether or ester bonds.[65,71,95]. Ferulic acid is often found in the form of feruloylated ω-hydroxyacids (sometimes with the later esterified to glycerol).[75,79,96] These structures have been suggested to connect both suberin domains.[58,95,96]

It is clear that available techniques are not sufficient to address these questions in further detail. Nevertheless, the study of suberin biosynthesis and the use of biochemical approaches such as reverse genetics may soon reveal new insights into suberin monomeric assembly.[58,63,101,102] Based on the knowledge obtained so far, several models have been proposed to illustrate the most logical assembly of monomers in native suberin. Figure 3 depicts two models of suberin structure as proposed by Bernards[58] and Graça[57].

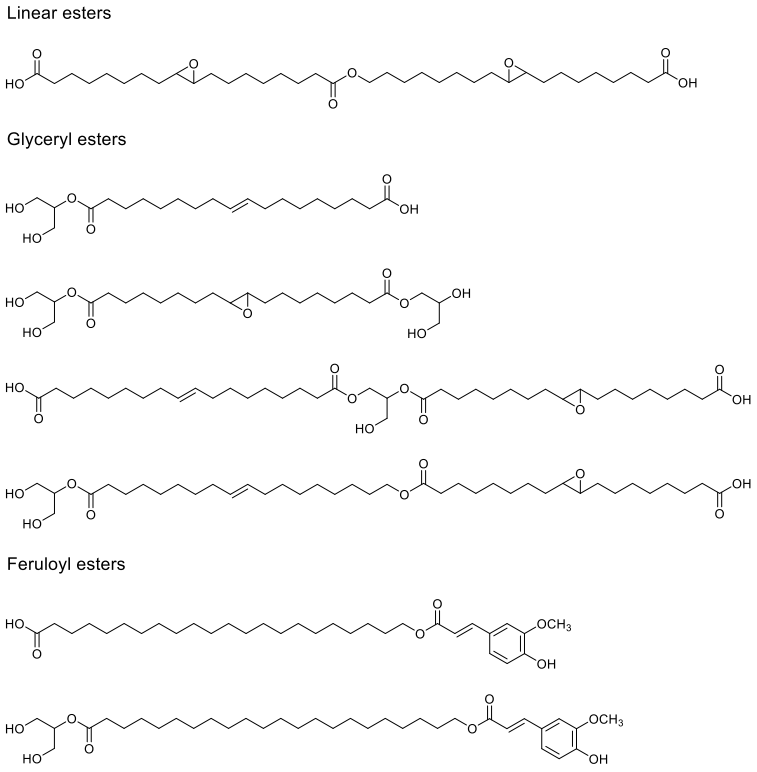


Figure 2| Suberin dimers and trimers.

4.4 Suberin Applications

Suberin has singular properties, highlighted by its physiological role in the plant, and therefore, suberin has inspired the design of new materials. Although only modestly exploited so far, suberin is often regarded as a promising source of monomers and oligomers for the synthesis of novel polymers.[52,103] Of particular relevance is the abundance of *mid*-chain hydroxy and epoxy fatty acids in suberin. These are rare in other renewable resources and difficult to synthesise chemically. In this point, birch outer bark suberin is particularly appealing since, when compared to that of cork, it displays a much more homogeneous composition, showing high abundance of ω-hydroxyacids, namely of 9,10-epoxy-18-hydroxyoctadecanoic acid[42,52]. Also relevant is the potential utility of enzymes involved in suberin biosynthesis for producing specific oxygenated fatty acids for specialty chemicals.[104]

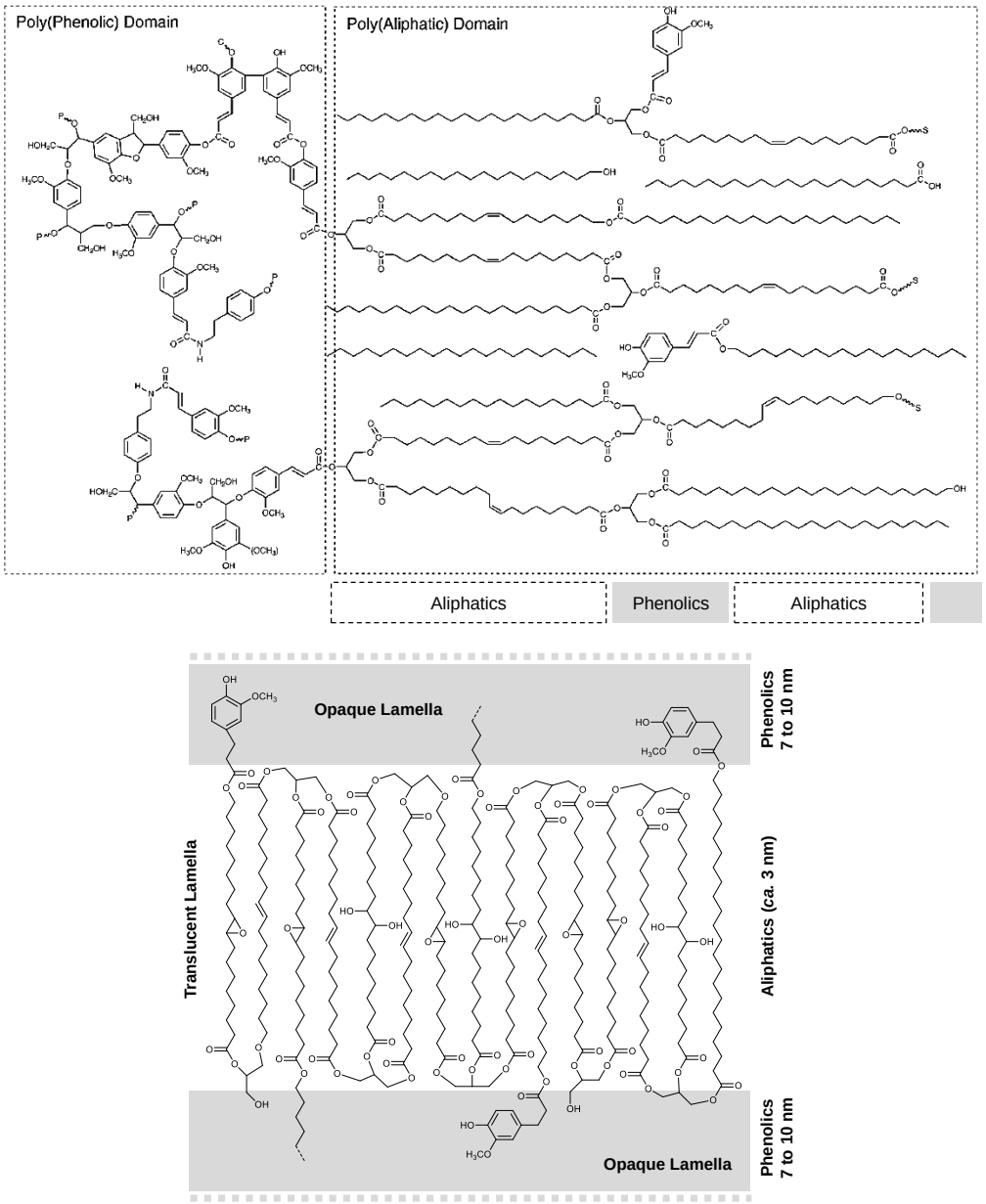


Figure 3| Tentative suberin models proposed by Bernards[58] (top; adapted from Pereira[46]) and by Graça (bottom; adapted from Graça *et al.*[57]). Top: the model includes a fraction of the suberin polyaromatic domain and two contiguous layers of aliphatics and associated phenolics. Linkages to other cell wall components are represented by letters: C, carbohydrates; P, phenolics; S, suberin. Bottom: the model represents a fraction of the suberin polyaliphatic domain, including the aliphatics and associated phenolics. The typical suberin lamellar ultrastructure observed by microscopic techniques is highlighted.[46,97]

Suberin monomers, as well as suberin-model monomers[105] have been used as precursors of new suberin-inspired materials, namely polyurethanes[106,107] and polyesters[105,108-110]. Monomers, obtained from extensive depolymerisation of birch outer bark and/or cork suberin, are re-polymerised either as obtained,[109] with co-monomers[106-108] or after isolation of specific monomers[110]. Polyesters are often prepared by common polycondensation or polytransesterification reactions,[105,108,109] however lipase catalysed[103,105,109,110] or microwave-based[105] methods have also been proposed.

Suberin has advanced towards elegant applications in skin care products (*e.g.* Suberlift™, Uplift Skincare® and Blackswan®) due to its tensor and smoothing effect.[111] This may well constitutes the only commercial application of suberin. Some reports also suggest the potential use of suberin as an additive in printing inks,[112] an absorbent of carcinogens[113] and as a natural antimutagenic agent[114].

Despite out of the focus of this thesis, the similarities between suberin and cutin are evident and cannot be ignored. Cutin is a glycerol-based biopolyester deposited in the outer surface of the epidermal cells.[64] Similarly to suberin, huge endeavour has been placed on the study of its monomeric composition and macromolecular assembly,[64] biosynthesis,[49,61,102] as well as on the preparation of cutin mimicking polymers involving either model monomers[115] or cutin depolymerisates[116].

5. Extractives: triterpenoids

Plants produce an outstanding diversity of metabolites which can be isolated by simple solvent extractions. These low molecular weight compounds are not covalently bound to the cell wall (non-structural elements) and constitute the so called plant extractives. The composition of these fractions is highly variable, even within the same species, and depends on factors such as climate and soil conditions, tree age and biotic or abiotic stresses.

In cork, extractives typically account for *ca.* 5 to 15 wt%[42,55,59,83,117-120] and are mostly composed of triterpenoids. Among these, friedelin is the dominant compound followed by betulin, betulinic acid and cerine (Figure 4). Non negligible amounts of

phenolics and waxes (including alkanes, alkanols and fatty acids commonly found as suberin monomers) are also commonly detected. Comparably high amounts of extractives, *ca.* 20 to 35 wt%, are also found in birch outer bark.[41,42,60,121] However, while in cork the extractive fraction shows a high heterogeneity, in birch outer bark this fraction is essentially composed of a single compound, betulin (*ca.* 80% of the extract weight). Other triterpenoids, namely betulinic acid and lupeol, are commonly co-extracted along with betulin, nevertheless this enriched extracts have significant value for commercial exploitation (Figure 4).[39]

As one can recognise, either cork either birch outer bark contain high contents of biologically active triterpenoids, a property that is consistent with the physiological function of these tissues in the plant, *i.e.* an acquired defence against plant pathogens.[39,122] The negligible commercial value of both biomass residues together with the indisputable value of triterpenoids and the difficulty to synthesise them chemically, has encouraged the extraction of these compounds from plant. Triterpenoids, a large and structurally diverse group of natural products, are derived from triterpenes (C_{30} structural combinations of isoprene units) and comprise one or more oxygen-containing functionalities such as alcohols, aldehydes, ketones and/or acids. The interest in this family of plant metabolites arises mostly from their wide spectrum of biological activities, such as anti-inflammatory, antiulcerogenic, antimicrobial, anticarcinogenic and antiviral (including anti-HIV) properties (see references[123] and references therein). As a consequence, these compounds have been growingly regarded as pharmaceuticals and cosmetics[123]. Some of these compounds are already used due to their cytostatic properties and/or reduced secondary-effects and many are under clinical trials.[123] Others show toxicity or improvable therapeutic potential and therefore the development of derivatives with enhanced properties is on-going.[123] During the last decades, Hill and Connolly[124,125] have repeatedly reviewed the scientific literature regarding triterpenoid natural occurrence, isolation and identification. References to the use of plants with high triterpenoid contents in traditional-medicine[126,127] further highlight the potential effects of these compounds some of which are increasingly inspiring the design of new drugs.[123,128]

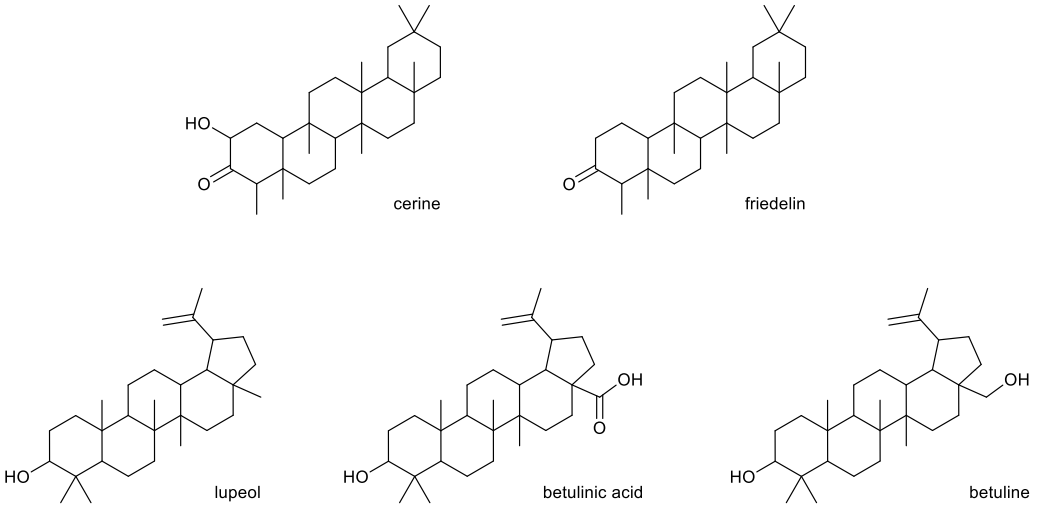


Figure 4| Triterpenoids typically detected in the extractive fraction of cork and birch outer bark.

6. Ionic Liquids

6.1 Ionic liquids: a neoteric class of chemicals

The first description of an ionic liquid goes back to the end of the 19th century when in 1888 Gabriel and Weiner reported the physical properties of ethanolammonium nitrate (melting temperature = 52-55 °C).[129] This field of chemistry remained somehow frozen until two decades ago and since then, ionic liquids have been increasingly attracting the attention of academia and industry. Significant progresses have been attained leading to the application of ionic liquids at industrial scales in a large number of processes.[130] Companies such as BASF, Exxon Mobil, BP, Institut Français du Pétrole and Degussa are some of the main playmakers at this level.[130]

According to the commonly accepted definition, ionic liquids are organic salts - thereof solely composed of ions - which are liquid at temperatures below a conventional temperature of 100 °C. These neoteric solvents comprise organic cations, usually quaternised aromatic or aliphatic ammonium, and either organic or inorganic anions (Figure 5). In addition, either the cation or the anion must be bulky or unsymmetrical as to avoid solidification.[29,131]

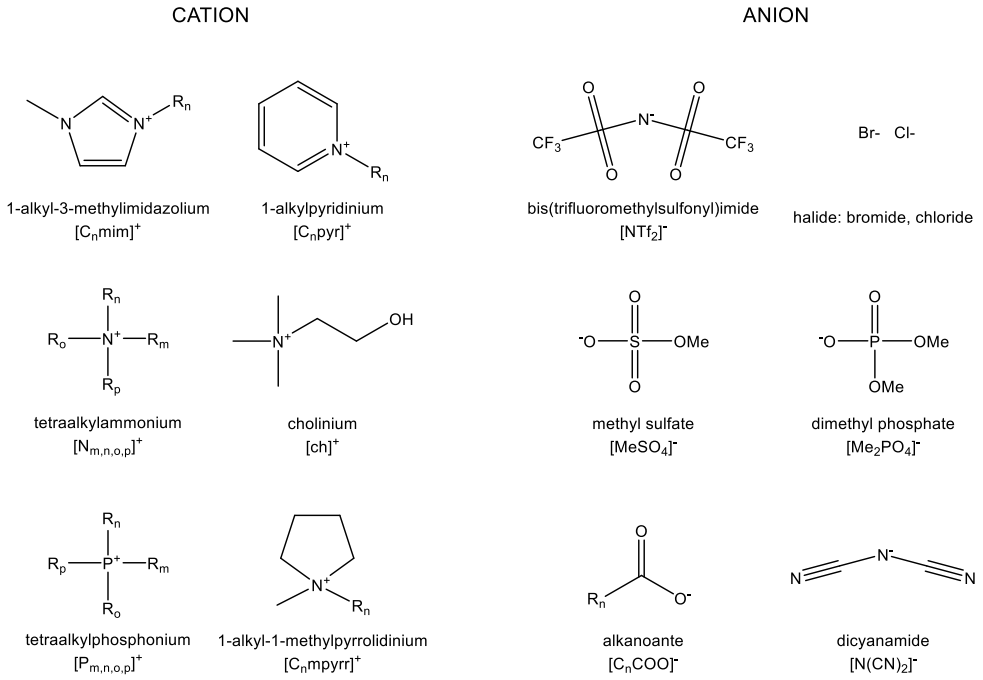


Figure 5| Cations and anions commonly used in ionic liquids.

Ionic liquids are often regarded has environmentally friendly and safe due to their negligible vapour pressure[31] and general non-flammability, chemical and thermal stability and recyclability. However these common generalisations are totally misleading since these properties should be considered for each ionic liquid and in the context of its application.

Truly remarkable, is the versatility of ionic liquids synthesis and handling, which is the basis for a clear sighted research and for the growing number of applications. In fact, ionic liquids can be designed and/or fine-tuned through alterations in the cations and/or the anions to address very specific thermo-physical, chemical and biological properties.[132-134] The range of cation and anion combinations is so vast, *i.e.* millions theoretical possible combinations,[130] that the number of ionic liquids exploited to this point represents just a very tiny illustration of their potential.

Another notable property of this neoteric class of solvents is their outstanding solvation ability, a property that arises from their tailored design and from the

combination of Coulomb, van der Waals, and specific interactions (typically, H-bonding) within a single compound[135]. Ionic liquids are able to dissolve (at least partially) a wide range of polar or nonpolar, organic or inorganic compounds,[135] providing new ways to carry out chemical reactions or industrial separations.[130] It was recently reported in different molecular simulation studies[136] that neat ionic liquids exhibit medium-range ordering, in other words, there are persistent microscopic domains in the liquid phase. Other simulation studies[137] on the microscopic dynamics of ionic liquids have also pointed out their slow dynamics and the persistence of local environments, typical of the glassy state. The segregation in polar and nonpolar domains at a nanoscale level in ionic liquids has changed the way in which solvation in these liquids is understood. Some solutes dissolve preferentially in low electrical charge-density domains, while others prefer the Coulomb environment of high electrical charge density regions, whereas some can even "be dissolved" at the interface between these polar and non-polar territories.[138] These facts account for the extraordinary versatility of ionic liquids as solvents when compared to common organic solvents or inorganic salts. Moreover, the interplay of Coulomb and dispersion forces in a single compound provides ionic liquids a whole new chemistry and allows them to act simultaneously as sophisticated extraction solvents, reaction media and/or catalysts.

To present, dialkylimidazolium-based ionic liquids constitute the most commonly studied ionic liquids family. This can be attributed to their inherent physical properties, such as low melting points and low viscosities, thermal stability, but also to their commercial availability at reasonable prices. However, when accounting for toxicity and environmental persistence it becomes evident that these solvents do not fully address the important Principles of Green Chemistry. True greenness should consider the extant eco-toxicological knowledge,[139-141] synthesis sustainability, handling and disposal risks,[142] the ionic liquid stability during function[143] as well as its environmental decay[140] (see Principles of Green Chemistry)[21,144,145]. Therefore, ionic liquids conscious design and the use of structure-activity relationships are essential tools to deliver safer formulations with enhanced technical performance.

In this scenario, improvements in the ionic liquids field led to "greener" alternatives such as the use of ammonium, phosphonium or sulphonium cations. The selection of benign cations, such as cholinium, constitutes, still today, one of the most important advances towards their conscious design.[146] This observation is highlighted by the fact that the quaternary ammonium salt, cholinium chloride, is classified as a provitamin in Europe and widely used as animal feed supplement.[147] Cholinium chloride environmental sustainability is also made known by the synthesis process: a one-step solventless reaction of hydrogen chloride, ethylene oxide and trimethylamine. Similarly the design of benign ionic liquids,[148] incorporating (*inter alia*) amino acids,[149] carboxylates[150-152] or glucose[153] has also been reported.

Due to the abovementioned properties, ionic liquids are inherently appealing media and they are often pointed as substitutes of common organic solvents. Again, this type of statements should be avoided. It is unquestionable that ionic liquids display unique properties although their conscious use should be taken into account. This new class of solvents should be regarded as an extension to the already existing possibilities in chemistry, namely in those processes where common solvents and catalysts failed or displayed only limited performance.[19] In this particular aspect, ionic liquids have proven unexpected opportunities in chemical synthesis,[30] catalysis,[154] electrochemistry,[155] materials sciences,[156] engineering fluids,[157-160] biomass dissolution[161-163] and at the interface of chemistry and life sciences in enzymatic catalysis[164] or as biologically active pharmaceuticals[133].

6.2 Ionic liquids for biomass processing

The solvent and catalytic ability of ionic liquids has not gone unnoticed to biomass processing. Many promising applications using these multifunctional solvents have been developed, ranging from the dissolution of raw biomass to the downstream depolymerisation of the ensuing products. In this scope, one mandatory example is the dissolution of cellulose in ionic liquids,[161,165-168] a demonstration that triggered tremendous interest in this area. Formulations coupling imidazolium or pyridinium cations with basic anions, such as acetate, phosphates and halogenates, have been

identified as the most appropriate for this task.[161,166-168] In fact, the superior solubility of cellulose in these ionic liquids is strongly correlated with the hydrogen bonding acceptor ability of the anions, as emphasised by their Kamlet-Taft parameters. Accordingly, nuclear magnetic resonance[169] and molecular dynamics studies[170,171] showed that these anions strongly interact with the hydroxyl groups of cellulose, disrupting its intra- and intermolecular hydrogen bond network.[161,162] Whereas the role of the anion is clear, the role of the cation remains dubious. Studies agree that cation certainly plays a role, however different interpretations have been proposed. These diverge from the absence of specific interactions between the cation and cellulose, to significant van der Waals attraction[162,172] or even the formation of covalent bonds[161,173]. Yet, the most accepted theory is that the main function of this ion is to solvate the anion-cellulose complexes, shielding the negative charges.[161] One should not disregard that cellulose dissolution is highly sensitive to water presence in the extraction media. In limited amounts it can promote dissolution by aiding cellulose hydrolysis whilst, at higher concentrations it may act as an anti-solvent or strongly interact with the ionic liquid impairing cellulose solubility.[162] One significant exception is the dissolution of cellulose at room temperature in tetrabutylphosphonium hydroxide and tetrabutylammonium hydroxide containing 40 to 50% water by weight.[174]

Upon dissolution in ionic liquid media, cellulose fibres are deconstructed into individual chains. Regenerated samples display therefore lower crystallinity (cellulose II) and polymerisation degrees when compared to their pristine forms (cellulose I), a feature that renders cellulose more amenable to chemical or enzymatic conversion into fermentable sugars, chemicals or biofuels[161,162]. These chains can also be subsequently derivatized in homogeneous conditions or simply reconstituted by precipitation with an antisolvent, *e.g.* water, acetone or acetonitrile.[161,162,168,175] Ionic liquids have been also used as reaction media for the conversion of carbohydrates into platform chemicals such as furfural and 5-hydroxymethylfurfural[176].

Equally notorious is the dissolution of lignin in these salts. This promising source of aromatic structures is fairly more complex and recalcitrant than cellulose, involving

extensive crosslinking through ether, aryl-ether and aryl-aryl bonds. As with cellulose, lignin dissolution is mainly controlled by the anion and strictly correlated with its hydrogen bonding basicity. Although this parameter does not need to be as high as for cellulose since some mid-range basic ionic liquids can also dissolve lignin. Accordingly, imidazolium methyl sulphate, acetate or chloride has been commonly reported as the most appropriate ionic liquids for lignin dissolution.[162,177] This process certainly involves partial depolymerisation of lignin *in situ* structure, a requirement that is corroborated by the suggestion that the anions catalyse or undergo nucleophilic attack to the β-O-4 linkages.[162,177,178] Similarly to cellulose dissolution, the role of the cation is not fully understood. Whereas some authors report the existence of π-π interactions between lignin and aromatic cations,[177,179,180] others mention this is not an essential parameter.[177] Nevertheless, it is accepted that these π-π interactions hamper the removal of solubilised lignin structures from the ionic liquid media.[177,181] Therefore, enormous amounts of water are usually necessary to perform this task.[162]

The complete depolymerisation and conversion of ionic liquid dissolved lignin has been proposed.[162,177] However the recalcitrance of this biopolymer makes it particularly resistant to chemical attack hampering progresses in this field. Accordingly, most of the studies have focused on lignin model compounds, namely in the catalytic disruption of *β*-O-4 linkages, the dominant chemical bond found in lignin. These reactions comprise mainly proton catalysed hydrolysis and catalytic oxidative reactions.[177] Interestingly, the presence (or even the addition) of limited quantities of water in the ionic media seems to have a beneficial effect either in lignin dissolution or chemical depolymerisation. This observation is likely attributed to the fact that water stimulates the hydrolytic fragmentation of ether bonds, nevertheless this parameter remains to be studied in detail.[166,182-184] Remarkably, recent studies demonstrated that benign ionic liquids, namely cholinium amino acid-based ones, can selectively dissolve and extract lignin from plant matrices even in the presence of significant amounts of water.[185-187]

To date, a variety of raw lignocellulosic biomass residues has been pre-treated and/or dissolved in ionic liquids. Wood[175,179,180,188], rice straw,[185,186] and swichgrass[189] constitute some examples. The regenerated lignocellulosic material often comprises a mixture of cellulose, hemicellulose and/or lignin. These results have triggered the development of selective precipitation methods to fractionate the dissolved components.[168,175,188]

An extremely heterogeneous group of other plant metabolites and biomacromolecules has also been isolated from natural matrixes using ionic liquids. These include starch,[190] zein,[190] chitin and chitosan,[191] keratin,[192] bioactive compounds,[193] vegetable oils,[194] fatty acids[195] and triterpenes[196] amongst many others. Another remarkable example is the production of biodiesel from vegetable oils in ionic liquid media.[194] These constitute extensively vast fields of research and thus they will not be described in further detail.

It is unquestionable that ionic liquids provide a new platform for the pre-treatment, dissolution, extraction and/or conversion of biomass components. Nevertheless, and similarly to biorefineries, this field is still at an early stage and better fundamental understanding of the involved mechanisms is required. Many challenges are envisaged, most of them arising from the ionic liquid. Both ionic liquid and biomass are usually moderately to strongly hygroscopic. Thus, moisture tolerance should be considered since, depending on its amount, it may halt dissolution. As the current methods to recover the dissolved components rely on the addition of an antisolvent, the ionic liquid recyclability will certainly require energy-demanding evaporation steps.[161,162,168] The thermal and chemical stability of ionic liquids is in general highly overestimated. This aspect has been largely neglected across the field and should be considered in the context of the process conditions. Moreover, formulations showing strong hydrogen bond basicity (incidentally those ones more appropriated for biomass dissolution) undergo thermal degradation, even at moderate temperatures due to the high reactivity of the anions.[162,197] Careful investigation of the stability of ionic liquids under processing conditions is therefore required. After a number of cycles, decomposition

products, carbohydrates, lignin fragments, inorganic salts and other solutes released during biomass processing are likely accumulated in the ionic liquid, hampering its performance and stability.[162,168] Accurate evaluation of the ionic liquids toxicity, biodegradability and environmental fate is highly recommendable.

7. Concluding Remarks

The ascribed ability of ionic liquids to disrupt biomass recalcitrant networks and to strongly interact with solutes suggested that cork might be also soluble in tailor-made ionic liquids. These constitute the core observations that initially triggered the development of suberin and betulin extraction methods using ionic liquids.

Apart from the publications included in this thesis only one report addressing suberin dissolution in ionic liquids is available in the literature. Mattinen *et al.* reported the solubilisation of enzymatically isolated and extractive-free suberin obtained from potato (*Solanum tuberosum* var. Nikola) using 1-allyl-3-methylimidazolium chloride.[198] Similarly, to date few studies addressed the extraction of triterpenes or triterpenoids using ionic liquids. Of particular interest in the framework of this thesis, is the study reported by Ressman *et al.*[196] addressing the microwave assisted extraction of betulin with 1-ethyl-3-methylimidazolium acetate. However, despite the good extraction yields and betulin high purity, this process presents significant drawbacks, namely the degradation of the ionic liquid.

In this thesis, the extraction of **suberin** and **betulin** from **cork** powder and **birch outer bark**, two industrial plant residues, is presented. In Chapter II it is described the extraction process of suberin from cork using ionic liquids. From this study, cholinium hexanoate was selected as the most appropriate formulation. Suberin extracted from birch outer bark and from cork was characterised, as described in Chapter III, showing an aliphatic and cross-linked nature. Remarkably, in Chapter IV, it was demonstrated that the mechanism of suberin depolymerisation by the ionic liquid evolves mainly through acyl-glycerol ester bond hydrolysis. This results in partial preservation of suberin structure, a feature that revealed to be essential for the preparation of suberin films, as

reported in chapter V. Finally, in chapter VI a microwave assisted extraction of betulin from birch outer bark using limonene as solvent is presented.

As a cross-linked biopolyester, suberin cannot be dissolved without depolymerising, at least partially, its structure. For the sake of simplicity, along this thesis the term "suberin dissolution" was used referring to both processes: depolymerisation and concomitant dissolution of the ensuing suberin structures. Accordingly, along this thesis, the suberin extracted by cholinium hexanoate is referred to be partially depolymerised and cross-linked. In this scope, the term "cross-linking" was used to emphasise the partial preservation of suberin structure which most likely retains, at least partially, its native cross-linked/esterified structure exhibiting molecular weights that are high enough to render them insoluble in common organic solvents. It is however recognized that, by definition, the dissolution of highly cross-linked macromolecular structures is not possible.

One final comment regarding what should be named suberin is necessary. As aforementioned, debate persists whether the term suberin should encompass either polyaromatic and polyaliphatic domains, or only the latter. For the sake of clarity, in the following Chapters the term suberin is used as described in the beginning of Section 4 of this chapter.

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Chapter II

Dissolution of cork biopolymers in biocompatible ionic liquids

The author contributed to the planning and execution of all the experiments described in this chapter, except the biological tests. The author also contributed to the analysis of the data and to the preparation of the manuscript.

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1. Abstract

Classically, the best attempts to separate suberin from cork biopolymers have been attained by conventional hydrolysis and methanolysis processes; here, we report a class of biocompatible and biodegradable cholinium-based ionic liquids, the cholinium alkanoates, which show a highly efficient and specific dissolution of suberin from cork.

2. Communication

Worldwide, the annual production of cork, which is the external bark of *Quercus suber* L., is 300 000 tonnes, half of which forms the basis of the Portuguese cork manufacturing industries.[1] Cork is a remarkable biocomposite, showing a very specific combination of properties, such as elasticity, compressibility, low permeability for liquids, and significant chemical/microbial resistance;[2] there is thus a significant interest in suberin (its major component) as a valuable source of property-enhancing additives[3]. The conventional pre-treatment of cork[4] removes the soluble components (extractives), but leaves an essentially insoluble matrix (extractive-free, or refined, cork) whose main components are polysaccharides, lignin and suberin (~20, ~30 and ~50 wt %, respectively).[5] Suberin is a complex cross-linked polymer, composed of aromatic and aliphatic domains.[6] The former domain is relatively similar to lignin, and possesses a very complex structure comprising units of hydroxycinnamic acid and, to a minor extent, monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols); the latter domain is composed mostly of units of C_{16}-C_{26} hydroxyacids that are primarily linked, *via* ester bonds, to glycerol.[2,7] This means that, structurally, cork has a strong relationship with lignocellulosic materials.

Ionic liquids exhibit a set of unique and astonishing properties, such as negligible vapour pressure, bulk non-flammability, and high thermal and chemical stability. Because their thermophysical and chemical properties can be fine-tuned through slight alterations to the cations, anions or both, they can address very specific requirements.[8] A major industrial interest in ionic liquids is the dissolution and processing of cellulose, which has been successfully achieved with some 1,3-dialkylimidazolium ionic liquids.[9,10]

The basis of their outstanding solvation behaviour results from their Coulombic environment,[8] and is thought to be due to an ionic liquid's ability to disrupt intermolecular hydrogen-bonding networks and interact with the hydroxyl groups of cellulose.[9,11] However, the scope of possible applications of 1,3-dialkylimidazolium ionic liquids is restricted due to their cost, toxicity[12] and/or environmental persistence due to the non-biodegradability[13] of the imidazolium ring.

Refined cork has been reported to be insoluble in many common solvents,[3] but its apparent similarities with other lignocellulosic materials suggest that it might be soluble in tailor-made ionic liquids. Here, we report the first example of refined cork dissolution by ionic liquids. Moreover, the ionic liquids selected are both biocompatible and biodegradable.

Learning from both the previous experience of the dissolution of lignocellulosic materials, and the extant toxicological and biodegradation data of ionic liquid cations and anions, a test group of ionic liquids was selected by combining three different cations (1-ethyl-3-methylimidazolium, $[C_2mim]^+$, 1-butyl-3-methylimidazolium, $[C_4mim]^+$, and cholinium, $[N_{111}C_2H_4OH]^+$) and six different anions. The effect of the anion was studied focussing initially on chloride, ethanoate and lactate anions, and, in the case of the cholinium ionic liquids,[14,15] on a further three alkanoates.

Cork samples were initially ground to a fine powder, and the soluble cork extracts (extractives) were removed as previously described by Gil *et al.*[4] Prior to the dissolution tests (all performed in triplicate), the ionic liquid and the refined cork powder were vacuum dried in order to remove water. The ionic liquid (*ca.* 1.6 g) was added to the refined cork powder (*ca.* 0.025 g) (ionic liquid:cork ≈ 2:1 v/v) and the mixture held at 100 ºC (which is below the degradation temperature of all the ionic liquids) without any stirring for 4 h. The dissolution was then stopped by adding an excess of de-ionised water (*ca.* 20 cm^3), and the insoluble residue was recovered by filtration and dried. Under these conditions, in separate experiments, no significant decomposition was detected for either the cork or the ionic liquids.

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy has successfully been used for cork characterisation, and consequently it has been

selected here for qualitative assessment of the dissolution process.[2,5,6] These studies determined quite precisely the infrared absorption features of the primary components of refined cork:

Suberin: 2921, 2852, 1737, 1242, 1158 and 724 cm^{-1}

Lignin: 1511, 855 and 819 cm^{-1}

Polysaccharides: 1092 and 1034 cm^{-1}

The samples of refined cork powder prepared for this study, both before and after heating (control), correspond exactly to these literature data.

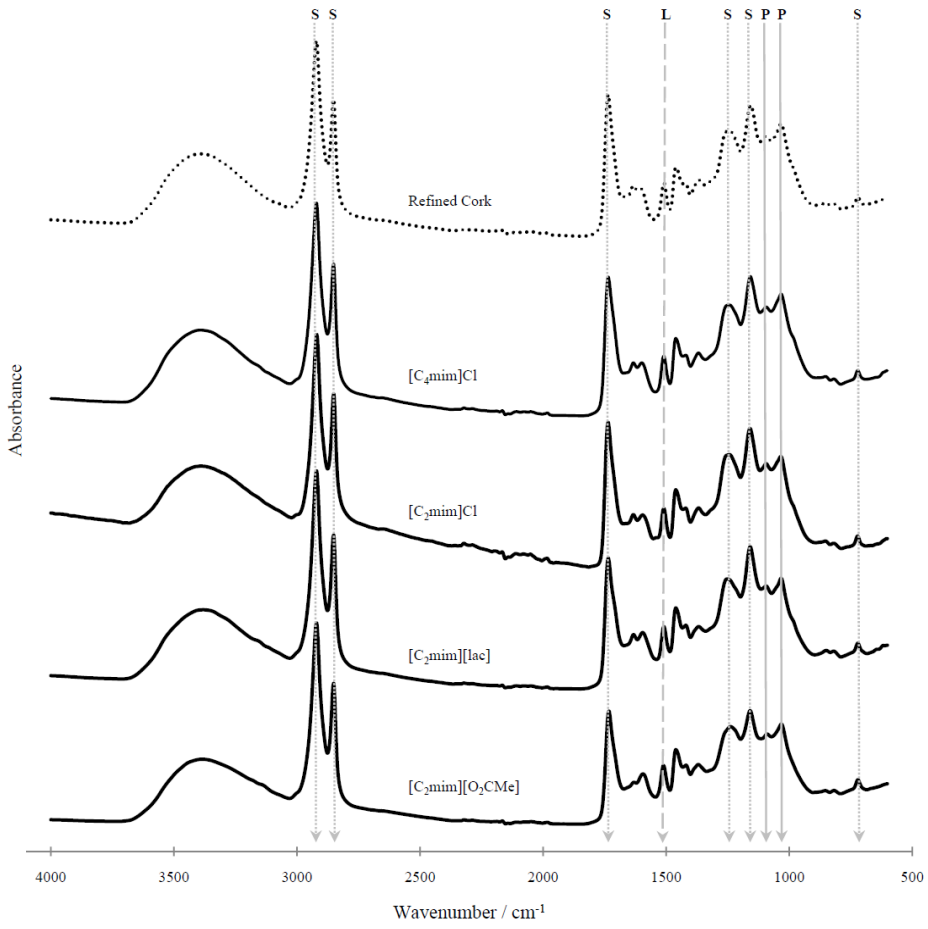

Figure 1| ATR-FTIR spectra of the insoluble cork fraction after treatment with different 1,3-dialkylimidazolium ionic liquids. The vertical lines identify the peaks mainly assigned to suberin (S), lignin (L) and polysaccharides (P).

ATR-FTIR analyses of the cork residue after its extraction by ionic liquids from the 1,3-dialkylimidazolium family are presented in Figure 1. Neither $[C_2mim]Cl$ nor $[C_4mim]Cl$ were able to dissolve significant amounts of the refined cork, even though they have been previously observed to enable significant dissolution of polysaccharides in lignocellulosic composites.[11,16] However, replacing the chloride with various carboxylate anions, namely lactate, $[lac]^-$, and ethanoate, caused the dissolution efficiency to increase significantly. The latter was more efficient and led to a greater dissolution of suberin (Figure 1, shown by the decreasing intensity of the peaks at 1737, 1242 and 1158 cm^{-1}). Both anions led to a small solubility of the polysaccharides, as illustrated by the changes in the intensity of the peaks at 1092 and 1034 cm^{-1}.

The results for the cholinium, $[Me_3NCH_2CH_2OH]^+$ or $[N_{111}C_2H_4OH]^+$, salts are presented in Figure 2 and Table 1. The cholinium ethanoate showed, relative to the 1,3-dialkylimidazolium ionic liquids, a significant increase in the dissolution of the refined cork. The lack of efficiency of the lactate anion, relative to the ethanoate (cork weight losses of 20.7 and 39.7 %, respectively), is unsurprising, as the lactate anion is less basic than the ethanoate anion. Further support for this simple concept is that the cholinium alkanoates (ethanoate, butanoate, hexanoate), showed augmented dissolution efficiency due to the increasing basicity and chain length of the anion (Table 1). The efficiency of the anions can be ranked as follows:

$$[lac]^- < [O_2CMe]^- < [O_2CPr]^- < [O_2CCHMe_2]^- < [O_2CC_5H_{11}]^-$$

Table 1| Data on the dissolution efficiency of refined cork in a range of cholinium alkanoates, $[N_{111}C_2H_4OH]Y$. The pK_a values of the conjugate acid of the anion of each ionic liquid is also presented.

Y^-	E_{Solv} (%)[a]	STD[b]	pK_a (HY)
$[O_2CMe]^-$	39.7	2.5	4.76
$[O_2CPr]^-$	44.1	10.8	4.84
$[O_2CCHMe_2]^-$	55.1	5.8	4.83
$[O_2CC_5H_{11}]^-$	64.9	7.9	4.85
$[lac]^-$	20.7	4.1	3.86

[a] Dissolution efficiency, E_{solv} (%) = 100 × ($m_{cork,\ t=0} - m_{cork,\ t=4h}$) / $m_{cork,\ t=0}$
[b] STD = standard deviation

The dissolution efficiency tracks the pK_a values of the conjugate acid of the anion, chloride being the least efficient. This goes in line with the dissolution of cellulose in ionic liquids, where the disruption of the hydrogen-bond network is also associated with the basicity of the anion.[17]

The dissolution of cork by $[N_{111}C_2H_4OH][O_2CMe]$, $[N_{111}C_2H_4OH][O_2CPr]$, $[N_{111}C_2H_4OH][O_2CCHMe_2]$, and $[N_{111}C_2H_4OH][O_2CC_5H_{11}]$ resulted, progressively, in a remarkable reduction (Figure 2) of the intensity of the peaks assigned to suberin (2921, 2852, 1735, 1242, 1158 and 724 cm^{-1}) in the insoluble cork residue. The peak assigned at 1735 cm^{-1} (associated with the carbonyl stretch of the ester groups) was virtually absent in the case of hexanoate, with concomitant formation of a small shoulder at 1712 cm^{-1}. This effect is possibly associated with the formation of acidic groups resulting from suberin removal.[5]

To eliminate the possibility that dissolution was due to hydrolysis of the ionic liquid, control tests were performed using the conjugate acids of the ionic liquids' anions. Butanoic and hexanoic acids (pure and dried with molecular sieves) were tested, resulting in a weight loss of 10.3 % and 17.2 % respectively, and no evident alterations were observed in ATR-FTIR spectra.

The cholinium family of ionic liquids present a very low toxicity,[18] especially to some eukaryotic organisms (*e.g.* ref [19]), due to the benign nature of the cation and its high biodegradability potential.[20] The toxicity (*i.e.*, growth inhibition effect[19]) of $[N_{111}C_2H_4OH][O_2CPr]$, $[N_{111}C_2H_4OH][O_2CCHMe_2]$, and $[N_{111}C_2H_4OH][O_2CC_5H_{11}]$ towards *P. corylophilum*, which has been previously demonstrated to show a moderate susceptibility to several ionic liquids,[19] were evaluated here. They exhibited very low inhibitory capacities, with MIC values of 150, 250 and 62.5 mM, respectively. The biodegradation of the most efficient system was then evaluated under aerobic conditions. After four weeks of incubation, the residual concentration of $[O_2CC_5H_{11}]^-$ in *P. corylophilum* cultures was <12 %, as determined by liquid chromatography. The biodegradation of the anion was confirmed by ^{1}H NMR spectroscopy and shown by the disappearance of the anion peaks in the spectra relative to the cation.

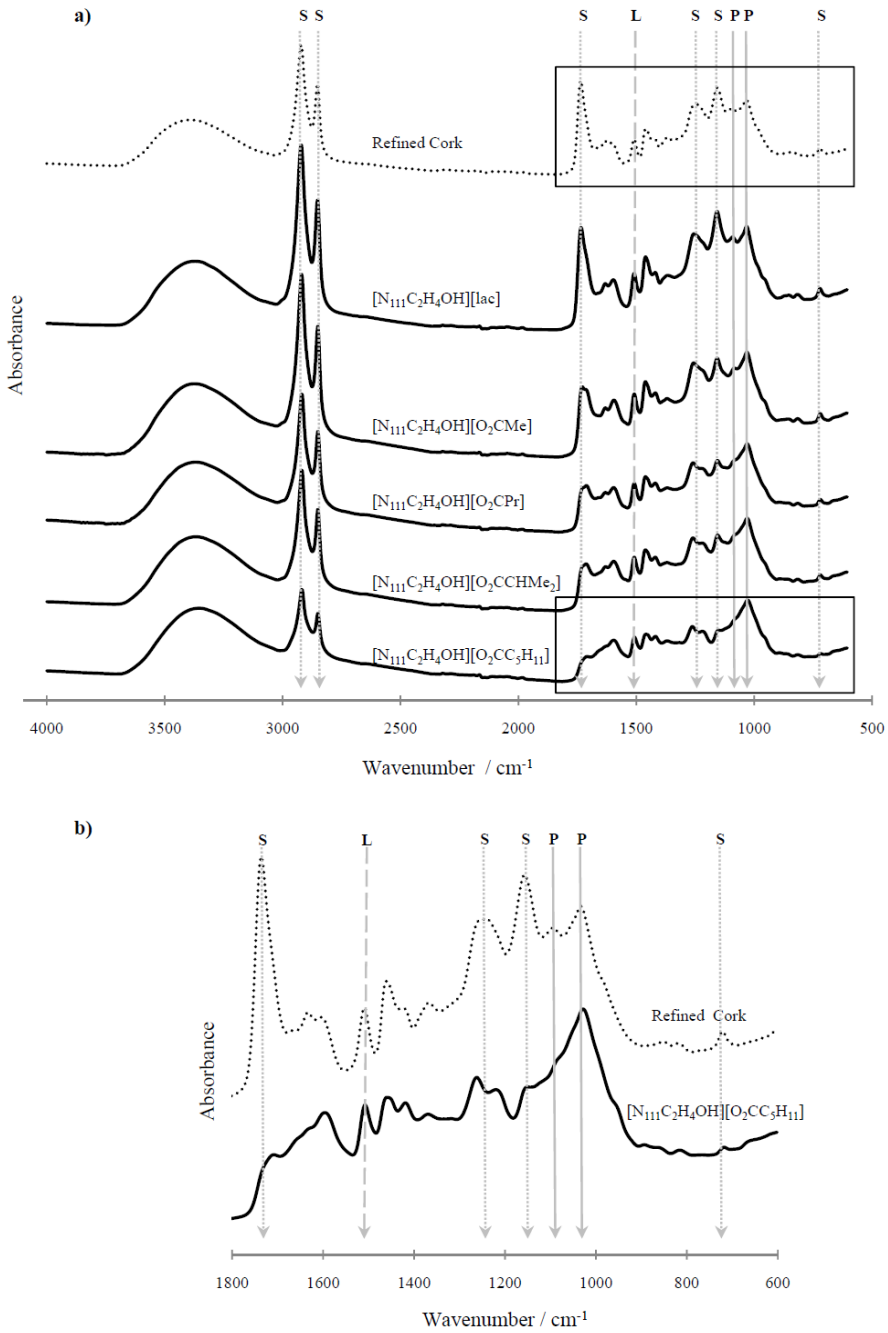

Figure 2| (**a**) ATR-FTIR spectra of the cork residue after treatment with different cholinium ionic liquids. (**b**) An expansion of the ATR-FTIR spectra of the cork residue after treatment with cholinium hexanoate. The vertical lines identify the peaks mainly assigned to suberin (S), lignin (L) and polysaccharides (P).

The biocompatible and biodegradable cholinium hexanoate appears to be the most promising ionic liquid for refined cork dissolution, particularly with respect to the separation of large quantities of suberin. Taking advantage of their synthetic tuneability,[8] there is clearly a huge potential for developing and exploring even more efficient systems.

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Chapter III

Suberin isolation process using cholinium hexanoate

Part 1: Suberin isolation from cork using ionic liquids: characterisation of ensuing products

Part 2: Isolation of suberin from birch outer bark and cork using ionic liquids

Chapter III

Part 1: Suberin isolation from cork using ionic liquids: characterisation of ensuing products

The author contributed to the planning and execution of all the experiments described in this chapter, as well as to the data analysis and to the preparation of the manuscript. SEM, EA, ^{13}C CP/MAS NMR, GC-MS and DMA analyses were performed by or in collaboration with technicians or co-authors.

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1. Abstract

Cholinium alkanoates, a class of benign ionic liquids, were demonstrated to efficiently extract suberin from cork. A detailed characterisation of the extracted material has yet to be attained. In the present study the significance of the alkylic chain length of the anion and the ionic liquid's basicity was investigated. The results obtained emphasise cholinium hexanoate's selection; it proved to be a straightforward process, also ensuring the recyclability and reusability of the ionic liquid. The extracted suberinic material has been thoroughly characterised for the first time by ATR-FTIR, NMR, GC-MS and thermal analyses. Data showed that it is mainly composed of oligomeric or polymeric aliphatic esterified structures, resulting from suberin partial cleavage. More than 40 wt % of the extracted suberinic material was found to be cross-linked. Even though, the composing monomeric units were similar to those usually identified in suberin samples obtained by the conventional extraction processes. These data pave the way for advanced studies of suberin monomers/oligomers as building-blocks for the development of novel biopolymers and biomaterials.

Keywords**:** cholinium hexanoate, cork, ionic liquids, renewable resources, suberin.

2. Introduction

Biomass feedstocks constitute a source of numerous value-added compounds, such as biopolymers, biofuels, and building-block chemicals.[1] Cork, the outer bark of *Quercus suber* L., is a remarkable plant composite displaying a very specific combination of properties, such as elasticity, compressibility, low density, low permeability, and significant chemical and microbial resistance.[2] Historically, cork utility goes back to the ancient Romans, and since then has been used essentially to manufacture stoppers and thermal/sound insulation materials. Globally, ≥ 300 000 tonnes of cork are processed *per annum* by industry, generating large amounts of residues (*ca.* 22 wt %), especially cork of small grains size, which, despite its interesting chemical composition, is generally burned to produce energy.[3]

Cork is composed of suberin, lignin, polysaccharides, and extractives (approximately 50, 20, 20 and 10 wt %, respectively).[4-6] Suberin, an aromatic-aliphatic cross-linked biopolyester, represents *per se* a source of property-enhancing additives.[7] It is a three-dimensional complex network occurring in the secondary plant cell wall. While its composition and native structural organisation are still controversial, the domains of suberin are generally thought to be arranged in a lamellar-type structure.[5,7-10] The aliphatic domain is composed mostly of even numbered units (C_{16}-C_{26}) of ω-hydroxyalkanoic and α,ω-alkanedioic acids (some with *mid*-chain unsaturation, epoxy or *vic*-diol functionalities), alkanoic acids and aliphatic alcohols. The aromatic domain shows a quite distinctive composition, with some similarities to lignin, predominantly composed of hydroxycinnamic acid units, with residual amounts of *p*-coumaryl, coniferyl, and sinapyl alcohols.[5,6,8,11-13] The suberin monomers are cross-linked *via* ester bonds involving glycerol units or aliphatic hydroxyl and carboxylic moieties.[12,14] However, the nature of the linking of suberin to the other cell wall domains remains uncertain.[15]

Depolymerisation of *in situ* suberin and its simultaneous isolation from the plant composite is traditionally a laborious process requiring harsh chemical processes.[7] These processes involve extensive ester bond cleavage, normally attained through alkaline methanolysis with sodium methoxide,[5,11,12,16,17] or alkaline hydrolysis.[18] Furthermore, suberin partial depolymerisation, leading to the formation of oligomeric structures, can be achieved using more gentle processes although with limited extraction yields, *e.g.* methanolysis catalysed by calcium oxide.[19]

The last few decades have witnessed an exponential growth of interest in ionic liquids – a disparate class of chemicals composed solely of ions that are liquid below a temperature conventionally defined as 100 °C.[20] Several hundred ionic liquids are already available and characterised,[21] and one can reasonably estimate that millions of cation/anion combinations are possible. Furthermore, fine-tuning of the cation and/or the anion might be used to address very specific thermophysical and chemical properties,[20] and even biological activity.[22] Ionic liquids usually exhibit a set of remarkable features, such as negligible vapour pressure,[23] bulk non-flammability, thermal stability, and high solvent ability.[20] The latter characteristic arises from the combination of the organic

functionalities of the ions with the Coulombic environment created by them, resulting in a structural arrangement of charged and apolar micro-domains.[24,25] A combination of the aforementioned properties with the simplicity of their synthesis and potential recyclability has led to the use of ionic liquids in various extant industrial applications.[21]

One of the most elegant examples of the industrial potential of ionic liquids was the demonstration that some imidazolium-based systems could successfully solubilise *in situ* cellulose.[21,26-28] This has been suggested to involve disruption of the intermolecular hydrogen-bonding network of cellulose.[27,29-31] More recently, other imidazolium-based ionic liquids have been shown to solubilise suberin isolated enzymatically from potato[32] and to extract lignin from lignocellulosic materials[33]. However, the recalcitrance to biodegradation of the imidazolium moiety,[34] together with its toxicity,[22] may restrict the large-scale application of these interesting observations.

In this context, there is no doubt that one landmark was the demonstration, by our group, that some cholinium alkanoates can efficiently extract *in situ* suberin from cork.[35] These ionic liquids have also shown to be both benign to eukaryotic organisms and biodegradable.[35,36] However, the detailed chemical and structural characterisation of the extracted suberinic material is yet to be attained. This constitutes the main goal of the present study and a key aspect to understand and improve the efficiency of this process. The suberinic materials isolated with cholinium hexanoate were characterised in terms of chemical composition, morphology, and thermal behaviour. Aiming to better understand the extraction process some alkanoates not considered in the previous study, were also investigated. The extracted suberinic material was found to be mainly composed of oligomeric and polymeric ester type structures.

3. Materials and Methods

3.1 Cork

Granulated cork was obtained from the cork producers Amorim & Irmãos SA (Stª Maria de Lamas, Portugal). The samples were ground to a fine powder (60 mesh) using a centrifuge mill (Retsch) and the cork extractives removed by sequential Soxhlet

extraction with solvents of increasing polarity (dichloromethane, ethanol and water) as previously described by Gil *et al.*[17] The extractives-free cork powder, hereinafter defined solely as cork, was further washed in an excess of deionised water for complete removal of low molecular weight compounds, and then dried prior to use.

3.2 Ionic liquids

The complete list of ionic liquids (Figure 1) used in this study is as follows: 1-ethyl-3-methylimidazolium hexanoate ($[C_2mim][O_2CC_5H_{11}]$); cholinium hexanoate ($[N_{111}C_2H_4OH][O_2CC_5H_{11}]$); cholinium octanoate ($[N_{111}C_2H_4OH][O_2CC_7H_{15}]$) and cholinium decanoate ($[N_{111}C_2H_4OH][O_2CC_9H_{19}]$). The cholinium alkanoate salts were synthesised by dropwise addition of the corresponding acid to aqueous cholinium hydrogencarbonate (Sigma ~80 % in water) in equimolar quantities, as described by Petkovic *et al.*[36] The 1-ethyl-3-methylimidazolium hexanoate was prepared using a similar method. First the chloride anion in 1-ethyl-3-methylimidazolium chloride was exchanged by hydroxide using an ion-exchange column (Amberlite® IRN-78). The resulting 1-ethyl-3-methylimidazolium hydroxide was then neutralised by an equimolar quantity of hexanoic acid. 1-Ethyl-3-methylimidazolium chloride of high purity was purchased from Sigma.

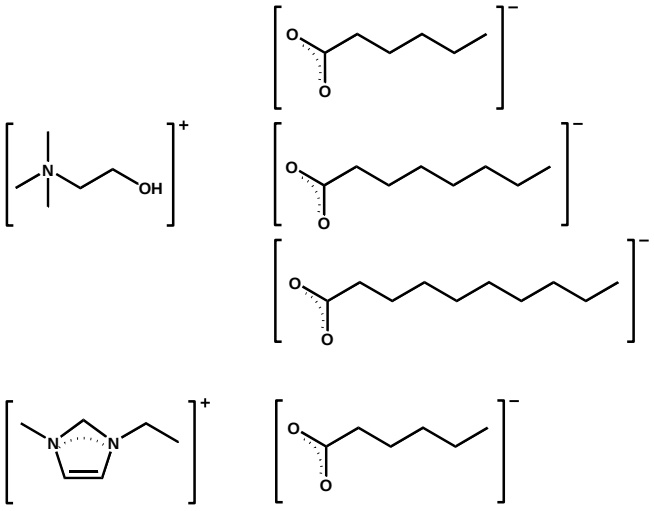


Figure 1| Chemical structures of the tested ionic liquids. From the top: cholinium hexanoate, octanoate and decanoate, and 1-ethyl-3-methylimidazolium hexanoate.

Ionic liquids purity was verified by ^{1}H and ^{13}C NMR spectroscopy at 25 °C, on a Brüker Avance III 400 spectrometer (Brüker BioSpin, Rheinstetten, Germany), and further confirmed by CHNS elemental analysis and electrospray ionisation mass spectrometry (ESI-MS) (Waters LCT Premier fitted with electrospray). The ionic liquids were dried prior to use by stir-heating *in vacuo* (40-70 °C, 24-48 h, *ca.* 0.01 mbar). The water contents, determined by Karl-Fischer titration, were below 0.5 wt %. The obtained salts fulfilled the requirements of the present study.

3.3 Other Chemicals

Ammonium nitrate ($[NH_4][NO_3]$), lithium nitrate ($Li[NO_3]$), dimethyl sulfoxide (DMSO), sodium hydroxide (≥97%), dichloromethane (99%), *n*-hexadecane (99%), decanedioic acid (99%), 12-hydroxydodecanoic acid (≥97%) and deuterated trichloromethane (99.8%) were purchased from Sigma.

3.4 Suberinic material extraction

The experiments followed the protocol previously described by Garcia *et al*.,[35] with some modifications (Figure 2). These aimed exclusively at facilitating and speeding-up the filtration step due to the large amount of suberinic materials being processed. Briefly, the ionic liquid was mixed with powdered cork (ionic liquid : cork ≈ 9 : 1 wt/wt) and kept at 100 °C during 4 h, with stirring (each in triplicate). At the end of the extraction process, DMSO was added to reduce the viscosity of the mixture,[27] facilitating its filtration through a 0.45 µm nylon membrane (Millipore, MA, USA). The cork insoluble residue was then washed thoroughly with an excess of water at 80 °C, and dried at 50 °C under a nitrogen purge until constant weight was attained. The ensuing filtrate, *i.e.* ionic liquid, the extracted material, DMSO and the water added to wash the cork insoluble residue, was kept at 4 °C for 1 h. This led to the precipitation of the extracted suberinic material, which was then recovered by centrifugation (30 min at 4 °C and 2450 *g*), washed twice with an excess of water to remove any remaining ionic liquid, and dried under a nitrogen flux, at 50 °C until constant weight was attained.

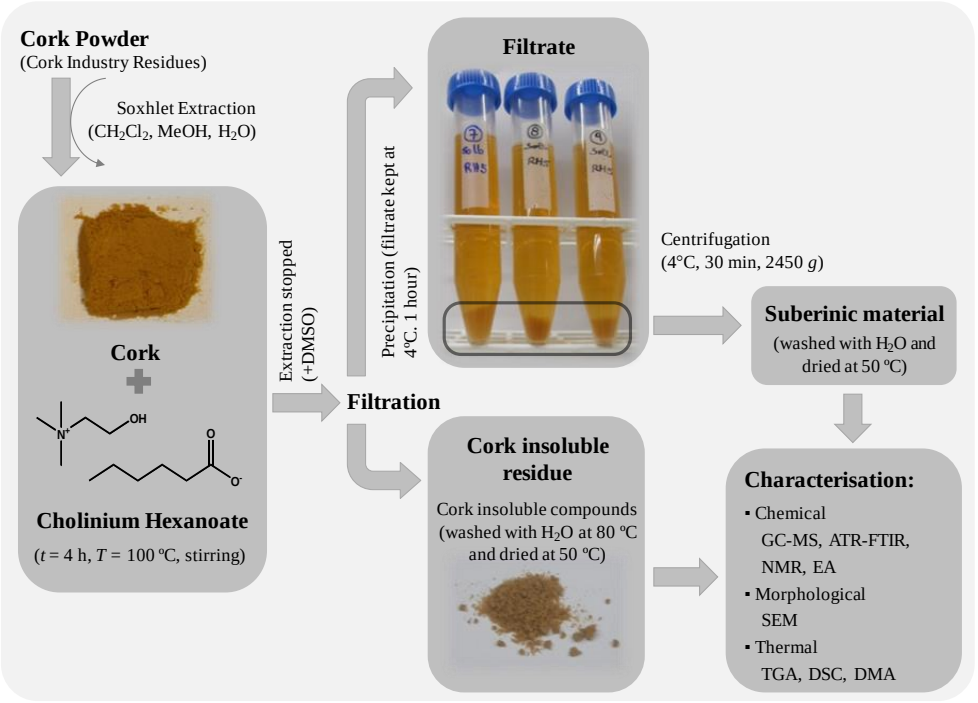


Figure 2| Schematic view of the scientific plan used in this study, where cholinium hexanoate is taken as an example.

3.5 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000-600 cm^{-1}, by accumulating 258 scans with a resolution of 8 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v5.0).

3.6 Scanning Electron Microscopy (SEM)

Samples were dried prior to use and coated with a thin layer of gold using a sputter coater (Polaron E-5100). Electron micrographs were recorded using an analytical field emission gun-scanning electron microscope (FEG-SEM: JEOL 7001F with Oxford light elements EDS detector) operated at 5 - 10 kV. The micrographs presented here were carefully selected, and are regarded to be representative of the different fractions.

3.7 Elemental Analysis (EA)

Elemental composition (C, H and N) was determined using a Leco TruSpec® Series elemental analyser. The oxygen (O) content was assumed to be the remaining amount of the sample and was calculated from the C, H and N composition.

3.8 Nuclear Magnetic Resonance Spectroscopy (NMR)

1D (^{1}H, ^{13}C) and 2D homo- and heteronuclear solution NMR spectra of suberinic materials were acquired on a Avance III 800 spectrometer (Brüker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a three channel 5 mm inverse detection probe head with pulse-field gradients along the *Z* axis. Spectra were run at 25 °C using standard Brüker pulse programs. ^{1}H and ^{13}C chemical shifts are referenced to trichloromethane. ^{13}C spectra were recorded at 201.24 MHz using the APT (attached proton test) sequence. ^{13}C Cross Polarization Magic/Angle Spinning NMR (CP/MAS NMR) spectra were recorded at 9.4 T on a Brüker 400 spectrometer using 9 kHz spinning rate and MAS with proton 90^{o} pulses of 4 μs. Chemical shifts are given in ppm from glycine. All NMR spectra were processed and analysed with MestreNova v. 6.0 (MestreLab Research S.L.).

3.9 Gas Chromatography-Mass Spectrometry (GC-MS)

A Trace GC 2000 Series gas chromatograph equipped with a Thermo Scientific DSQ II mass spectrometer was used. The GC-MS was first calibrated with pure reference compounds (12-hydroxydodecanoic acid and decanedioic acid), representative of the major classes of suberinic compounds, relative to *n*-hexadecane (internal standard). Compounds identification was based on the equipment spectral library (Wiley-Nist) and on previously published data based on their EI-MS fragmentation patterns and/or retention times.[11,13,18,37] Each sample was analysed by two complementary methods:

- Method 1, suberinic material was converted to the corresponding trimethylsilyl derivatives and analysed as previously described,[11] allowing identification of monomeric structures present in the mixture;

- Method 2, in order to analyse the composition of the oligomeric/polymeric fraction of suberinic material, samples were submitted to an alkaline hydrolysis step in order to release their monomeric constituents. Briefly, the samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v), at 95 °C, during 4 h.[38] The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1 M HCl, extracted three times with dichloromethane, and dried in a rotary evaporator. Finally, samples were trimethylsilylated as mentioned above, prior to GC-MS analysis.

3.10 Thermogravimetric analysis (TGA)

TGA data were obtained using a TGA-Q50 TA Instruments. All samples were run in crimped aluminium pans with pin-hole under a nitrogen atmosphere (100 cm^3 min^{-1}). Samples were dried *in situ* at 100 °C for 30 min and heated up to 600 °C, at a heating rate of 1 °C min^{-1}. Universal Analysis version 4.4A software was used to determine the degradation temperature ($T_{x\%,\,deg}$), onset temperature (T_{onset}), the weight of water adsorbed by the sample in equilibrium with atmosphere (wt_{H_2O}), the weight of the solid residue remaining at 600 °C ($wt_{600\,°C}$) and the derivative thermograms. $T_{x\%,\,deg}$ and T_{onset} are, respectively, defined as the temperature of a specific weight loss after the drying step, and as the intersection of the baseline weight after the drying step with the tangent of the weight *vs.* temperature curve as decomposition occurs. wt_{H_2O} is defined as the weight loss occurring since the beginning of the experiment until the end of the *in situ* drying step.

3.11 Differential Scanning Calorimetry (DSC)

DSC analyses were carried out with a DSC – Q200 TA Instrument. The DSC was calibrated for temperature and heat flow with indium samples and operated under constant purging of nitrogen (50 cm^3 min^{-1}). Samples were hermetically sealed in aluminium pans and heated/cooled up to 120/-80 °C at a constant rate of 5 °C min^{-1}, followed by a 5 min isotherm at 120/-80 °C. Three heating/cooling cycles were repeated. The first cycle was used to clear the sample thermal history. When the second and the third cycles were identical, the latter was used for data collection. The characteristic peaks were analysed using Universal Analysis, version 4.4A software. Melting

temperature (T_m) was determined as the minimum of the melting endothermic peak during the heating cycle.

3.12 Dynamic Mechanical Analysis (DMA)

DMA measurements were carried out with Tritec 2000 DMA Triton equipment operating in the bending (single cantilever) mode. Tests were performed at 1 and 10 Hz and the temperature was varied from -100 to 150 ºC at 2 ºC min^{-1}. A small amount of the powdered sample was dispersed in a foldable stainless steel sheet from Materials Pocket of Triton technology.

4. Results and Discussion

Cholinium hexanoate, a biocompatible and biodegradable ionic liquid, was demonstrated to promote a highly efficient extraction of the suberin from cork.[35] These findings were based on analyses of the IR absorption peaks of cork insoluble residues (which can be attributed to its specific constituents without significant error, Supplementary Section S1).[5,6,12,39] The efficiency ranking of the previously tested anions (*viz.* ethanoate < DL-lactate < butanoate ≈ *iso*-butanoate < hexanoate) suggested that the extraction process was controlled by the length of the anion alkylic chain and increases progressively with its basicity.[35] The alkaline requirement of the ionic liquid-based process for the extraction of suberinic materials from cork resembles conventional approaches where this is taken as a critical factor.[5,11,12,16-19,37] In fact, hexanoic acid alone was observed to be unable to extract suberin from cork.[35] In order to mimic the Coulombic component of an ionic liquid environment, eutectic mixtures of inorganic salts ($[NH_4][NO_3]$ + $Li[NO_3]$) were tested. These mixtures also failed to extract cork components (extraction yields < 5 wt %).

4.1 Extraction of suberinic materials from cork with alkanoate-based ionic liquids

Inspired by these initial findings, the extraction of suberinic materials by cholinium alkanoates carrying a long alkylic chain anion and high basicity was tested in the present study. These included the previously tested cholinium hexanoate,[35] and also cholinium

octanoate or decanoate, here tested for the first time. Their extraction ability was initially determined comparing untreated cork with cork treated with each of the ionic liquids, *i.e.* cork insoluble residue (Figure 3). Even though the basicity increases slightly with the length of the anion alkylic chain (hexanoate < octanoate < decanoate), cork mass losses and ATR-FTIR spectral profiles of the cork insoluble residues were comparable (Figure 3a). The ATR-FTIR spectra showed a remarkable reduction of the peak intensities attributed to suberin (2921, 2852, 1737, 1242, 1158 and 724 cm^{-1}). In addition, the polysaccharide and lignin domains in the cork insoluble residues remained apparently unaltered. Data make apparent that the tested cholinium alkanoates led to an extensive extraction of suberin from cork. In view of cork chemical variability, one can assume that the extraction yields obtained in this study, are comparable to the maximum yields reported for alkaline methanolysis of cork (~55 wt %).[12]

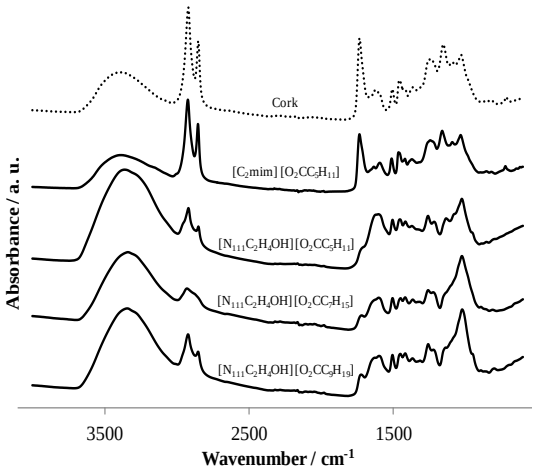
In the present study, it also became apparent that the cholinium cation *per se* plays an important role for suberin extraction. In fact, 1-ethyl-3-methylimidazolium hexanoate was unable to efficiently extract suberin from cork (extraction yields of 30.6 wt %, Figure 3a). The superior performance of the cholinium hexanoate, relative to the 1-ethyl-3-methylimidazolium hexanoate, is probably related with the strength of interaction between cation and anion. In fact, the carboxylate moiety of the anion might strongly interact with the protic hydrogen in the imidazolium ring, partially blocking the extraction of suberin from cork.

Petkovic *et al.* demonstrated that the minimal inhibitory concentration of cholinium hexanoate against fungi was significantly higher (by one order of magnitude) than those of cholinium octanoate or decanoate.[36] It is therefore irrefutable that cholinium hexanoate raises the greenness of this novel suberin extraction process.

4.2 Chemical characterisation of the extracted suberinic material

The superior efficiency of cholinium hexanoate, together with its high biocompatibility,[35,36] makes ultimate its selection for a deeper characterisation of the extracted material, *i.e.* suberinic material.

a)



E_{ext} / %[a]	STD[b]	pK_a (HY)
30.6	2.5	4.85
67.2	2.0	4.85
63.7	1.5	4.89
58.7	6.2	4.90

[a] Extraction yield:
E_{ext} / % = 100($m_{cork,\ t=0} - m_{cork\ insoluble\ residue}$) / $m_{cork,t=0}$;
[b] STD = standard deviation

b)

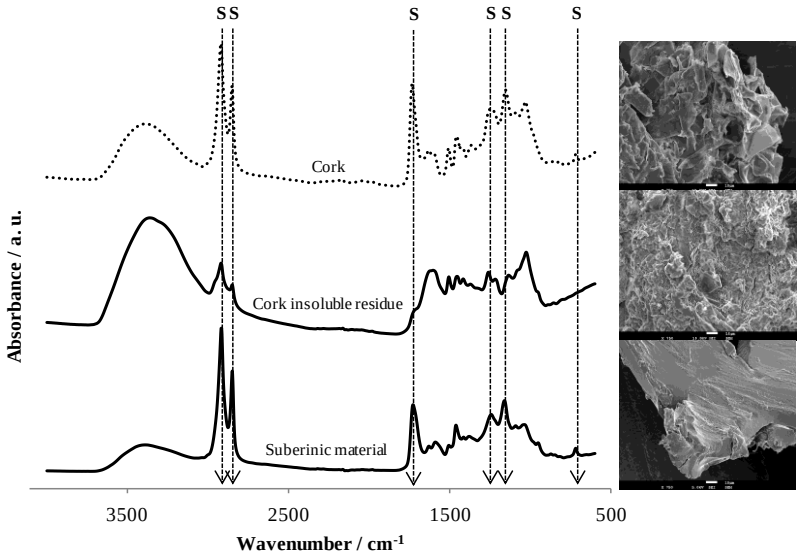


Figure 3| Analysis of the extraction of suberin from cork with the selected ionic liquids, namely 1-ethyl-3-methylimidazolium hexanoate, cholinium hexanoate, cholinium octanoate and cholinium decanoate. (**a**) ATR-FTIR spectra of cork insoluble residue. Side table shows suberin extraction yield after ionic liquid treatment and the pK_a of the conjugate acid (HY) of the corresponding anion. (**b**) ATR-FTIR spectra of the cork insoluble residue and the suberinic material after extraction with cholinium hexanoate. Side figures show the corresponding SEM images (magnification 750×). Vertical lines stand for peaks mainly assigned to suberin (S).

Samples of cork, suberinic material and cork insoluble residue were analysed by SEM (Figure 3b). This provided information on morphological alterations introduced in cork after extraction with cholinium hexanoate. The SEM images show that the cholinium hexanoate extraction process has substantially affected the morphology of powdered cork. A drastic destruction of the cork cell walls can be noticed in the cork insoluble residue. In addition, the suberinic material displayed a very homogenous morphology typical of non-structured material.

Elemental analysis showed that the C, H, O and N relative abundance in the suberinic material (Table 1) were similar to that of a suberin sample extracted through alkaline methanolysis.[13] As expected, due to the presence of long aliphatic chains, the suberinic material was more enriched in C and H when compared to the cork insoluble residue. Likewise, enrichment in hydroxycinnamic acid derivatives, *e.g.* feruloyltyramine,[40] might justify the increment in N relative content detected in the suberinic material. However, vestigial amounts of cholinium hexanoate might also have contributed to this increase both in the suberinic material and in the cork insoluble residue. The increment in the O relative content detected in the cork insoluble residue is most probably due to its enrichment in polysaccharides and lignin.

The ATR-FTIR spectrum of the suberinic material (Figure 3b) is dominated by major peaks at 2921, 2852 cm^{-1}, normally attributed to the long aliphatic chains of suberin.[5,12,13,39] In addition, the high intensity of the band at 1730 cm^{-1}, which is usually assigned to the vibration of carbonyl groups typical of esters, suggests that this material was extracted mainly in the esterified form.

Table 1| Elemental analysis of cork, cork insoluble residue, and suberinic material.

	C / wt %	**H / wt %**	**O / wt %**	**N / wt %**
Cork	61.90	7.41	30.14	0.55
Cork insoluble residue	55.20	6.37	36.17	2.26
Suberinic material	67.40	9.09	22.35	1.16
Suberin[13]	68.00	9.76	20.66	n.d.

n.d. – not determined

The ^{13}C CP/MAS NMR spectrum clearly demonstrates that the suberinic material (Figure 4) owns an essential aliphatic and esterified nature. In fact, the two major resonances at δ 30 and 33 ppm are attributed to methylenic carbons of typical long aliphatic carbon chains; and the resonance at around δ 173 ppm is assigned to carbonyl carbons of ester groups (Figure 4). The resonance at δ 148 ppm is usually assigned to quaternary carbons present in lignin-type structures.[6,17] However, it is difficult to discriminate if these quaternary carbons are the typical aromatic compounds of suberin, lignin or both. Other resonances at about δ 54, 64, 73 ppm and δ 130 ppm were also detected and are assigned to carbons nearby hydroxyl or ester groups and to vinylic carbons, respectively. Though these resonances are typical of suberin, one cannot disregard that they might also be associated with the presence of polysaccharides and lignin, respectively.

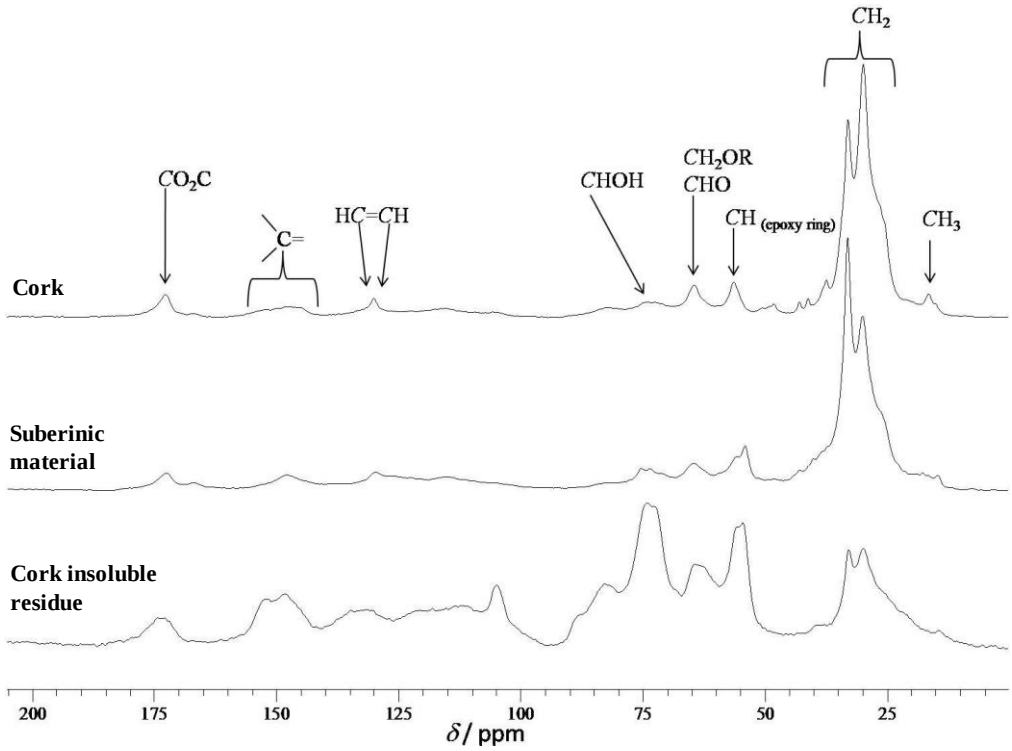


Figure 4| ^{13}C CP/MAS NMR spectra of cork, and both suberinic material and cork insoluble residue after extraction with cholinium hexanoate. R stands for H or ester group.

As expected, the ^{13}C CP/MAS NMR spectrum of the cork insoluble residue showed major resonances typical of polysaccharides and lignin at δ 54, 64, 73, 83, 105 ppm and δ 131-133, 148 ppm, respectively (Figure 4). Although of low intensity, some typical suberin resonances (at δ 30, 33, 173 ppm) were also detected (in accordance with the corresponding ATR-FTIR spectrum, Figure 3b). This seems to imply that suberin extraction from cork by cholinium hexanoate, though extremely efficient, was not complete.

The ^{13}C CP/MAS NMR data further validate the initial interpretation of elemental and ATR-FTIR analyses. The extracted material, which shows an essential aliphatic and esterified nature typical of suberin, could not be completely solubilised in organic solvents. The dichloromethane insoluble cross-linked fraction of the suberinic material represents 42 ± 2 wt %. This observation reinforces that despite the high extraction efficiency of cholinium hexanoate, this process took place by partial depolymerisation of suberin.

4.3 Chemical characterisation of the organic soluble fraction of suberinic materials

In order to complete the chemical characterisation of the suberinic material, the organic soluble fraction was further characterised by ^{1}H and ^{13}C NMR spectroscopy. These methodologies were combined with 2D COSY, HSQC and HMBC studies[41] for refining the spectral attributions (Supplementary Section S2). The data depicted in Table 2 include the list of the functional groups identified and their NMR assignment. The obtained ^{1}H NMR spectrum (Figure 5) is characterised by the presence of a major group of resonances, in the range δ 1.25-2.38 ppm, associated with suberin methylenic groups, in different chemical environments, namely in the long aliphatic chains and nearby ester groups. Two additional resonances at δ 4.05 and 4.82 ppm, assigned to methylenic and methinic protons directly linked to an ester group, were also observed. These features were confirmed by ^{13}C NMR analysis (Table 2) which showed dominant aliphatic carbon resonances at δ 25-35 ppm and at 173-190 ppm assigned to -COO- groups. Other minor resonances were also detected, namely those assigned to vinylic groups (^{1}H: δ 5.34 ppm; ^{13}C: δ 130 ppm), aliphatic methyl groups (^{1}H: δ 0.72-1.05 ppm; ^{13}C: δ 12 ppm) and

aromatic compounds (^{1}H: δ 5.92-8.09 ppm; ^{13}C: δ 100-150 ppm). The presence of aromatic protons (highlighted in the magnified section of Figure 5), confirms also the data observed in the ^{13}C CP/MAS NMR. Overall, the NMR data clearly suggest that the structural features of the suberinic material extracted by cholinium hexanoate are highly consistent with those previously reported for suberin extracted by conventional methods from *Q. suber* cork.[5,11,13,16,17,42] Importantly, using conventional methods only the organic soluble suberinic monomers and oligomers released during hydrolysis are extracted.[7]

Table 2| ^{13}C and ^{1}H NMR analysis assignments of the functional groups identified in the suberinic material.

^{13}C δ / ppm	^{1}H δ / ppm	Functional Group	Assignment[5,11,13,16,17,42]
12	0.72 – 1.05	CH_3	Aliphatic methylic groups
25 – 35	1.25, 1.29	CH_2	Aliphatic methylenic groups
—	1.53 – 1.67	CH_2CH_2CO; CH_2CH_2O	Methylenes in the β position to hydroxylic, ester and carboxylic groups
—	2.00	CH_2=CH-CH	Allylic protons
—	2.25 – 2.38	CH_2COO; CH_2COOH	Methylenes linked to carboxylic moieties
—	3.00	CH	Epoxy ring
62	3.64	CH_2OH; CHOH	Primary and secondary alcohol
52	3.66	OCH_3	Methoxy groups
54	4.05	OCH_2	Methylenes adjacent to ester groups
n.i.	4.82	OCH	Methine adjacent to ester groups
130	5.34	CH=CH	Vinylic groups
100 – 150	5.92 – 8.09	Ar	Aromatic signals
173 – 190	—	COO; COOH	Ester and carboxylic acid groups

n.i – not identified

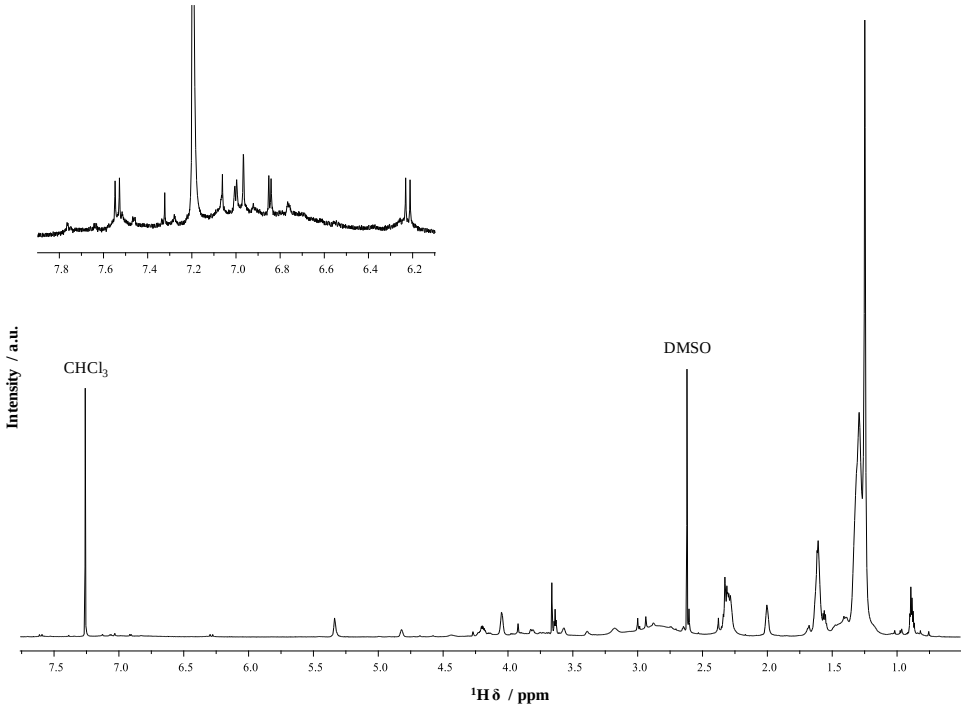


Figure 5| ^{1}H NMR spectrum (800 MHz, 25 °C) of the suberinic material fraction soluble in deuterated trichloromethane. The magnified section of the spectrum corresponds to its aromatic domain.

4.4 Chemical characterisation of the suberinic materials monomers

GC-MS analysis of the monomeric composition of the suberinic material (Method 1) led to the identification of only 3.9 wt % (Table 3). The low identification yield is certainly due to the fact that the suberinic material is mainly in the form of high molecular weight components, *i.e.* oligomeric or polymeric fractions of suberin-type structures. This reinforces the idea of its esterified nature, which is also compatible with the presence of insoluble cross-linked polyester type structures. The main families of monomeric compounds detected were extractives (not covalently bonded to cork), alkanoic acids and monoacylglycerols derivatives, even if each accounted for ≤ 1-2 wt %. Minor amounts of alkan-1-ols, hydroxyacids, alkanedioic acids and aromatic compounds were also identified (< 0.2 wt %).

As reported above, analysis of the suberinic material by GC-MS lead to a very low identification yield of monomers. In order to circumvent this, the GC-MS analysis

was repeated in a sample hydrolysed by alkaline hydrolysis (η = 62 wt %) prior to the silylation (Method 2, Table 3). After hydrolysis, the amounts of detected compounds in the suberinic material were considerably higher, accounting for 36 wt % (~13.5 wt % of cork). These yields are close to those previously reported.[7,13] In the hydrolysed suberinic material, a considerable increment on the content of typical suberin hydroxyacids (*e.g.* 22-hydroxydocosanoic and 9,10,18-trihydroxyoctadecanoic) and alkanedioic acids (*e.g.* 9,10-dihydroxyoctadecanedioic), as well as aromatic compounds (in particular ferulic acid), was observed.[7,11,37] Most monomeric compounds with ≥ 3 OH and/or COOH functionalities were only detected after hydrolysis of the suberinic material. Once more this is in accordance with its cross-linked and therefore insoluble nature.

Table 3| Main suberin monomers identified by GC-MS analysis of the suberinic material by Method 1 and Method 2, *i.e.* non-hydrolysed and hydrolysed samples respectively. Results are given in mg of compound *per* gram of suberinic material.

Identification	Method 1 $m_x/m_{suberin}$ mg/g	Method 2 $m_x/m_{suberin}$ mg/g
Alkan-1-ols	**1.04**	**7,44**
Octadecanol	0.03	0,11
Eicosanol	0.08	0.43
Docosanol	0.64	5.06
Tetracosanol	0.30	1.84
Alkanoic acids	**5.70**	**10.76**
Hexanoic acid[a]	4.70	1.43
Tetradecanoic acid	0.03	0.24
Hexadecanoic acid	0.24	2.35
Octadeca-9,12-dienoic acid (linoleic acid)	—	0.18
Octadec-9-enoic acid (oleic acid)	0.03	0.21
Octadecanoic acid	0.38	3.16
Eicosanoic acid	0.04	0.12
Docosanoic acid	0.27	3.07
Hydroxyacids	**1.75**	**191.28**
10-Hydroxydecanoic acid	0.05	0.41
16-Hydroxyhexadecanoic acid	—	1.72
18-Hydroxyoctadec-9-enoic acid	0.04	33.83
18-Hydroxyoctadecanoic acid	—	0.79
20-Hydroxyeicos-9-enoic acid	—	1.29
20-Hydroxyeicosanoic acid	0.03	4.11

Table 3| (continued)

Identification	Method 1 $m_x/m_{suberin}$ mg/g	Method 2 $m_x/m_{suberin}$ mg/g
22-Hydroxydocosanoic acid	1.43	68.27
24-Hydroxytetracosanoic acid	0.20	7.94
9,18-Dihydroxy-10-methoxyoctadecanoic acid[b]	—	8.87
9,10,18-Trihydroxyoctadecanoic acid	—	53.00
cis- Mid-chain,18-dihydroxyoctadec-9-enoic acid	—	3.25
trans- Mid -chain,18-dihydroxyoctadec-9-enoic acid	—	2.55
Mid -chain,18-trihydroxyeicosanoic acid	—	5.23
Alkanedioic acids	**0.43**	**49.71**
Hexadecanedioic acid	—	2.48
Octadecanedioic acid	tr	0.64
Octadec-9-enedioic acid	—	6.32
9,10-Dihydroxyoctadecanedioic acid	—	26.52
Eicosanedioic acid	0.22	1.83
9,10-Dihydroxyeicosanedioic acid	—	3.12
Docosanedioic acid	0.21	8.81
Aromatics	**0.35**	**30.48**
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	tr	0.65
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	0.20	1.31
3.4-Dihydroxybenzoic acid	0.08	—
4-Hydroxy-3-methoxy-cinnamic acid (*cis*-ferulic acid)	—	0.84
4-Hydroxy-3-methoxy-cinnamic acid (*trans*-ferulic acid)	0.06	27.68
Extractives	**20.50**	**44.29**
β-Sitosterol	1.47	2.63
Friedelin	9.76	10.14
Betulin	6.95	27.24
Betulinic acid	2.31	4.29
Monoacylglycerol derivatives	**8.57**	**0.00**
1-Monohexadecanoylglycerol	1.30	—
1-Monooctadecanoylglycerol	0.41	—
1-Monodocosanoylglycerol	1.18	—
1-Monotetracosanoylglycerol	1.20	—
1-Mono[docosanedi-22-oic acid-1-oyl]glycerol	4.48	—
Glycerol	**1.92**	**0.43**
Others	**3.18**	**24.90**
Other epoxy derivatives	—	24.90
n.i.	3.18	—
Total Identified Sample (wt %)	**3.87**	**35.79**

tr – trace amounts; n.i. – not identified; [a] Not accounted for compounds quantification; [b]Methoxyhydrin artefact from 9,10-epoxy-18-hydroxyoctadecanoic acid.[13]

4.5 Thermal characterisation of the extracted suberinic material

The thermal characterisation of the suberinic material was performed by TGA (Table 4, Figure 6a) and DSC analyses (Figure 6b). During the TGA analysis the samples were dried *in situ* showing weight losses (wt_{H_2O}) of 3.02, 5.76 and 2.01 wt % for cork, cork insoluble residue and suberinic material, respectively. Previous studies reported similar values for cork wt_{H_2O}.[43,44]

All the samples were observed to be thermally stable up to approximately 200 °C (Figure 6a). The suberinic material showed the lowest $T_{5\%,\,deg}$. This suggests the presence of a small fraction of volatile molecules, certainly including the suberin free monomeric units detected by GC-MS analysis. The thermal resistance of the suberinic material (T_{onset} = 310.7 °C) was similar to that of cork (T_{onset} = 301.8 °C), and much higher than that of the cork insoluble residue (T_{onset} = 229.6 °C). This agrees with previous reports on similar materials.[45] The close similarity of their decomposition profiles, together with their comparable T_{onset} values, underlines the key role of suberin in cork's high thermal resistance. Accordingly, the cork insoluble residue presented the lowest thermal resistance, certainly owing to its high polysaccharides content, which normally display T_{deg} < 200 °C.[2,39,46] Above 200°C and up to 450 ºC all samples showed a gradual multi-step weight loss, typical of complex biomass-based samples. The solid residue remaining at 600 °C accounted for 20.5, 36.1 and 16.5 wt % for cork, cork insoluble residue and suberinic material, respectively. Even though lower $wt_{600\,°C}$ would be expected for suberinic materials,[13] similar values have been observed in chemically re-polymerised suberin monomers.[47] Hence the high thermal stability of the extracted suberinic material is most probably due to its esterified nature and the presence of cross-linked polyester type structures. In addition, the high value of $wt_{600\,°C}$ for the cork insoluble residue is apparently due to its high content in lignin.[48]

The DSC thermograms of cork and suberinic material, displayed one broad melting transition that span for several tens of degrees (Figure 6b), ranging approximately from 30 to 70 °C. This behaviour is probably a consequence of the complex composition of these samples.

Table 4| Degradation temperature ($T_{x\%,\ deg}$) and onset temperature (T_{onset}). Weight of water adsorbed by the samples in equilibrium with atmosphere (wt_{H_2O}) and weight of the solid residue remaining at 600 °C ($w_{600\ °C}$).

	Cork	Cork insoluble residue	Suberinic material
$T_{5\%,\ deg}$ / °C	231.4	213.0	205.2
$T_{10\%,\ deg}$ / °C	262.6	239.8	257.4
T_{onset} / °C	301.8	229.6	310.7
$wt_{600\ °C}$ / %	20.46	36.12	16.48
wt_{H_2O} / %	3.02	5.76	2.01

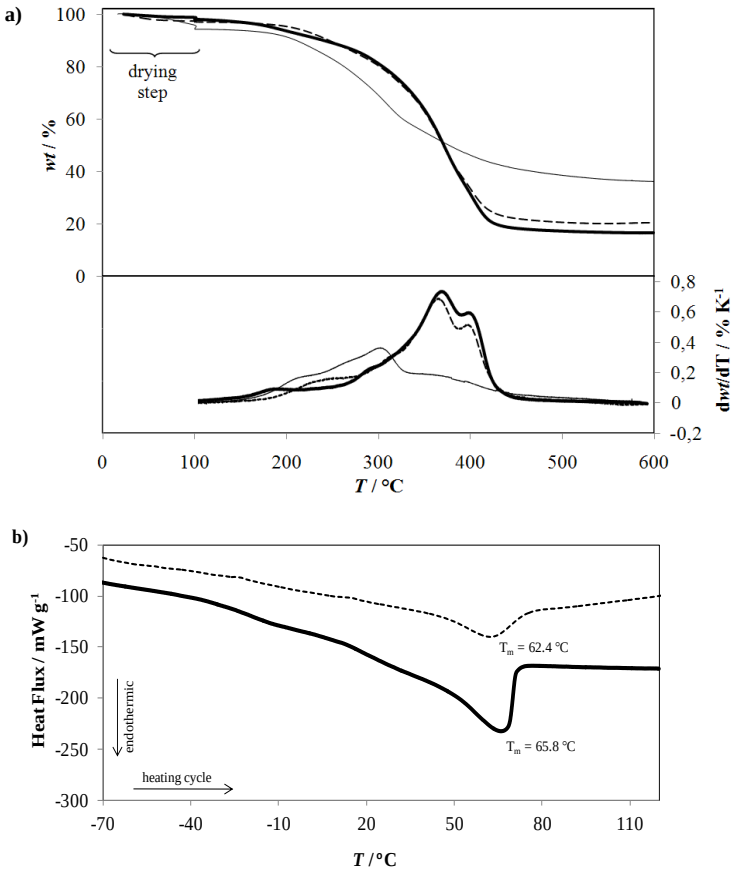


Figure 6| Thermal analyses: (**a**) TGA thermogram (top) and the first derivative of weight loss as a function of temperature (bottom). (**b**) DSC thermogram. (—) cork insoluble residue, (**▬**) suberinic material, (- - -) cork.

A true glass transition for suberin was not observed by DSC, even upon testing different heating/cooling rates (data not shown). Nevertheless, the glass transition for suberin, herein estimated by DMA (which reports higher sensitivity), was -51.0 °C, agreeing with previous reports.[45]

4.6 Environmental sustainability and ionic liquid recyclability

The sustainability of this extraction process can be ensured by optimal design of the filtration step in order to avoid the use of DMSO. Preliminary scale-up tests using cholinium hexanoate were performed (4 h at 100 °C with stirring). At the end of the extraction process, the mixture was immediately filtrated in a pressurised tank at *ca.* 80 °C in order to remove the cork insoluble residue. The ensuing filtrate was then diluted with water and cooled down to 4 °C, leading to precipitation of the extracted suberinic material. The extraction yield and composition (as issued from ATR-FTIR analysis of the materials) were similar to those reported above. The ionic liquid in the aqueous supernatant was recovered by eliminating the water under high vacuum conditions (*ca.* 0.01 mbar). The purity of the recovered ionic liquid was verified by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (Supplementary Section S3). The yield of cholinium hexanoate recovered by this method was greater than 99 %. When reused, and accounting for cork chemical variability, no significant loss of efficiency was observed, leading to suberin extraction yield of 58.3 ± 2.3 wt %.

5. Conclusions

The high potential of some cholinium alkanoates, having a long alkylic chain in the anion and high basicity, for extracting suberin from cork was investigated. Cholinium hexanoate showed excellent extraction efficiency and selectivity towards suberin, and high biocompatibility and biodegradability potential. Moreover, it could be easily recycled without loss of extraction efficiency. The chemical and thermal characterisation of the material extracted by cholinium hexanoate, *i.e.* suberinic material, was herein attained for the first time. The extracted material showed suberin typical features, with an aliphatic and esterified nature, and a high thermal resistance. The chemical analysis

before and after alkaline hydrolysis reveals that the isolated suberinic material was mainly composed of cross-linked aliphatic polyester type structures. It seems reasonable to assume that a better understanding of the suberin extraction from cork by cholinium hexanoate, and the detailed study of the structural features of the resulting materials, might hold new insights regarding suberin *in situ* structural organisation.

Importantly, this process can be easily applied to other suberin enriched sources, such as birch outer bark. Numerous applications for these new suberinic materials could be envisaged, *e.g.* macromonomers for the synthesis on novel materials.

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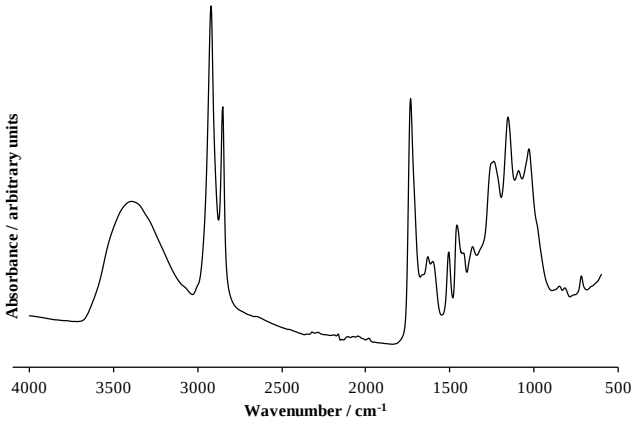
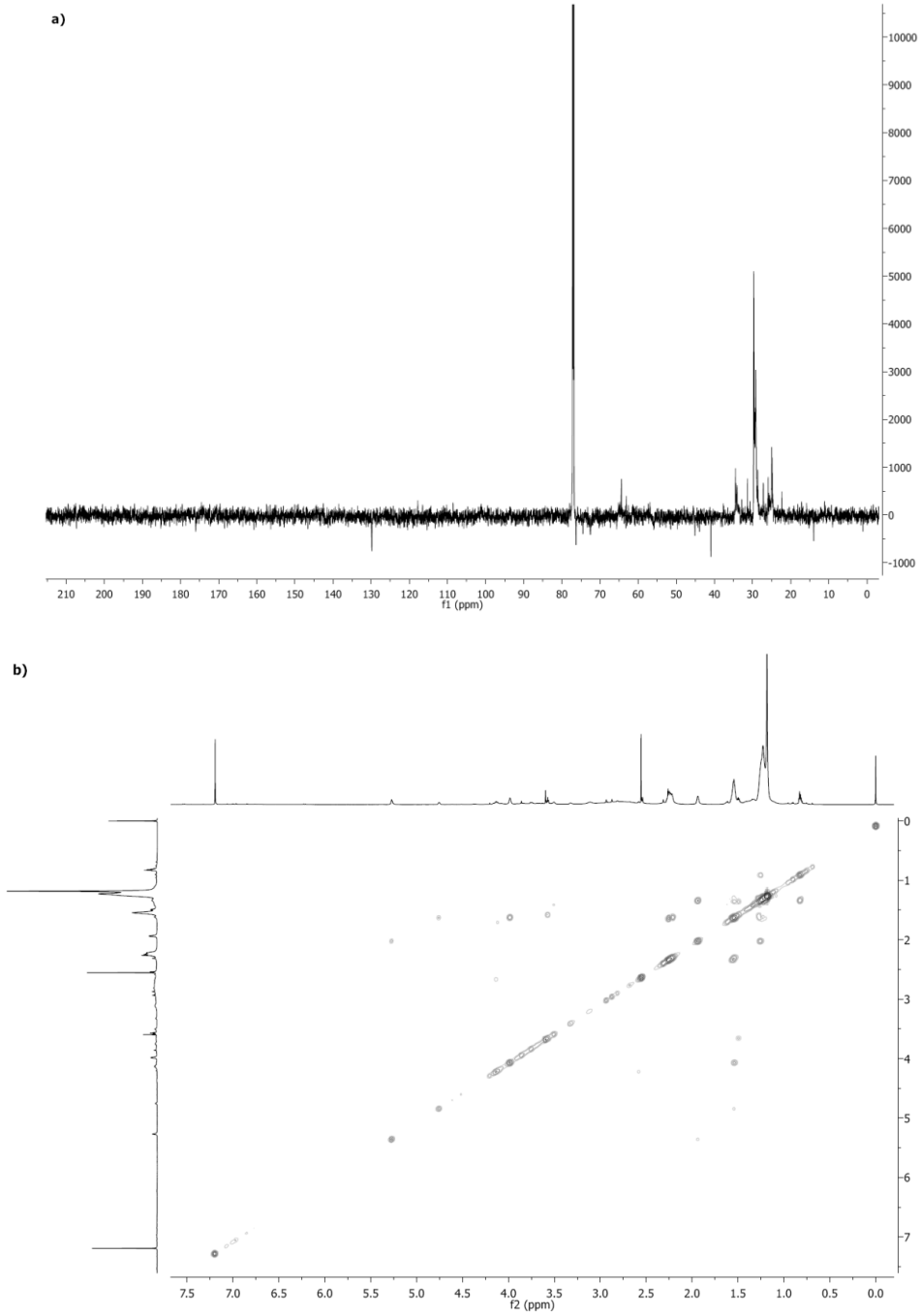
7. Supplementary Information

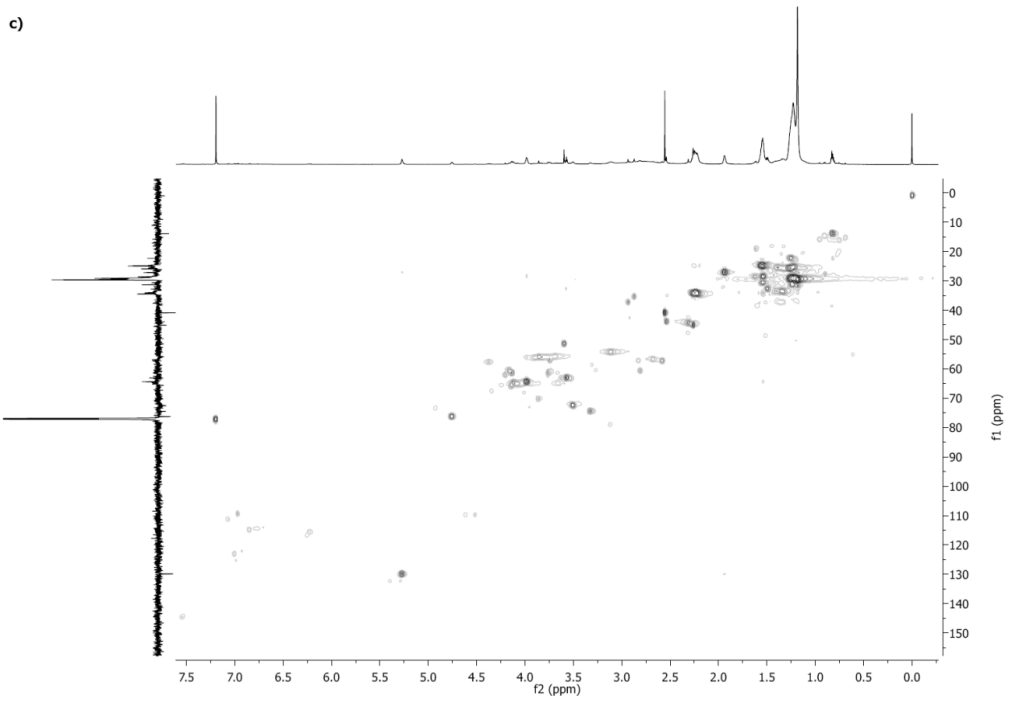
Section 1 (S1)| ATR-FTIR spectrum and assignment of cork major compounds (control),[5,6,12,39] treated under the extracted conditions (4 h at 100 ºC) in the absence of ionic liquid. The underlined cork constituent represents the major contribution to the infrared band.

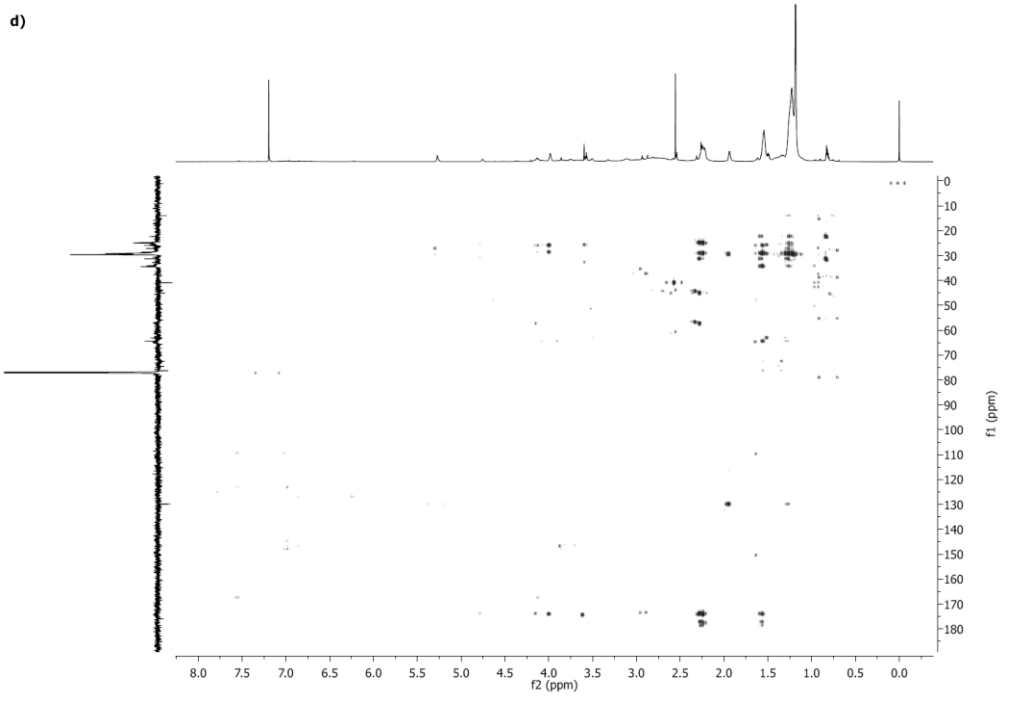


Wavenumber / cm^{-1}	Description	Assignment
3395	OH stretch	water and polissacharides
2921	CH aliph. stretch	suberin, polissacharides and lignin
2852	CH aliph. stretch	suberin, polissacharides and lignin
1737	C=O stretch (ester groups)	suberin, polissacharides and lignin
1635	C=C stretch	suberin
1606	C=C stretch	suberin and aromatic lignin
1511	C=C stretch	aromatic lignin (Guaiacyl-type)
1460	CH asym. deformation	suberin, polissacharides and lignin
1424	C-C stretch in the ring	small amount of aromatic ring Guaiacyl-type lignin
1366	CH sym. deformation	suberin, polissacharides and lignin
1242	CO stretch	suberin, polissacharides and lignin
1158	CO asym. stretch	suberin, polissacharides and lignin
1092	CH, CO deformation	polissacharides and lignin
1034	CH, CO deformation	polissacharides and lignin
855	CH out-of-plane deformation	aromatic ring Guaiacyl-type lignin
819	CH out-of-plane bending	aromatic ring Guaiacyl-type lignin
724	CH_2 rocking	suberin

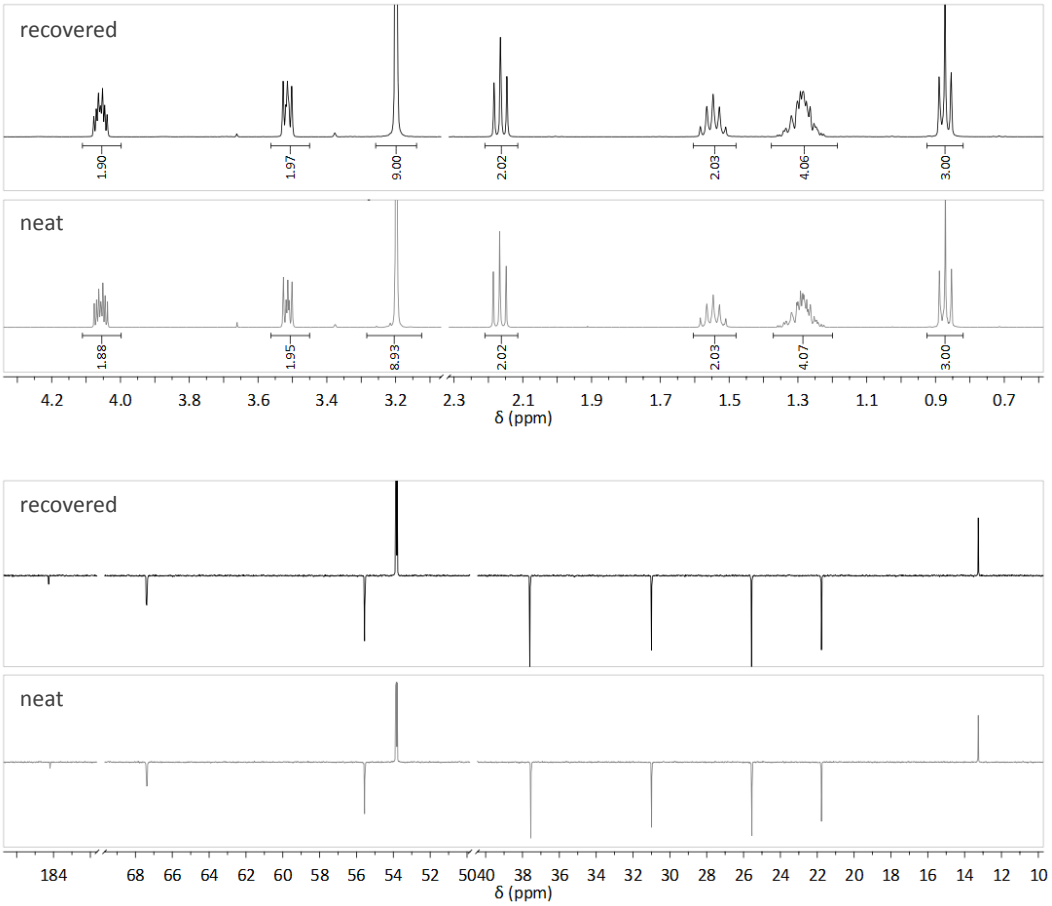
Section 2 (S2)| NMR spectra (800 MHz, 25 °C) of the suberinic material fraction soluble in deuterated trichloromethane: **a**) ^{13}C NMR, **b**) COSY, **c**) HSQC and **d**) HMBC.




c)
f1 (ppm)
f2 (ppm)


d)
f1 (ppm)
f2 (ppm)

Section 3 (S3)| ^{1}H NMR (top) and ^{13}C APT NMR (bottom) spectra of cholinium hexanoate as recovered by water evaporation, compared to those of the neat ionic liquid. Both samples were analysed in D_2O.



ESI-MS recovered cholinium hexanoate:

Calculated for $C_5H_{14}NO^+$ $[M]^+$: m/z = 104.11
Found 104.5
Calculated for $C_6H_{11}O_2^-$ $[M]^-$: m/z = 115.08
Found 114.9

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Chapter III

Part 2: Isolation of suberin from birch outer bark and cork using ionic liquids

The author contributed to the planning and execution of all the experiments described in this chapter, as well as to the data analysis and to the preparation of the manuscript. ^{13}C CP/MAS NMR and GC-MS analyses were performed by or in collaboration with technicians or co-authors.

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1. Abstract

Cholinium hexanoate, a biocompatible and biodegradable ionic liquid, was recently demonstrated to efficiently and selectively extract suberin from cork, combining high extraction efficiency with isolation of a partial depolymerised material. In the present paper, we report a comparative study of the characterisation of suberin extracted from birch outer bark and from cork using cholinium hexanoate. It became apparent that both extracted suberin samples showed still a cross-linked nature, *i.e.* likely to be closely related to *in situ* suberin. Suberin samples were mainly constituted by oligomeric or polymeric structures in turn essentially composed of long chain hydroxyacids monomers. Their high thermal stability together with the oligomeric/polymeric nature, open new perspectives for suberin use as macromonomers in the development of bio-based polymeric materials. This also contributes for the valorisation of suberin rich agro-forest residues.

Keywords**:** suberin; cork; birch outer bark; cholinium hexanoate ionic liquids.

2. Introduction

Knowledge on natural polymers, such as starch, cotton, proteins and wool, is ancient and remounts to the beginning of the Human History.[1,2] During the last decade, we have been witnessing a renewed and exponential increase of interest in the production of chemicals, materials, fuels and energy obtained from renewable resources. This is especially true in the so-called biorefinery concept,[3,4] which involves, in many cases, application of valuable components from by-products of agro-forest industries, such as suberin from cork residues.

Suberin, a complex aromatic-aliphatic cross-linked biopolyester, is widespread in the plant Kingdom but it is particularly abundant in *Quercus suber* L. cork (30-50 wt%) and *Betuta pendula* outer bark (40-50 wt %).[5-10] This hydrophobic biopolyester plays a key role as a protective barrier between the plant and the environment.[11] Suberin constitutes a major natural source of valuable compounds such as ω-hydroxyacids,

α,ω-dicarboxylic acids and corresponding *mid*-chain epoxy or dihydroxy derivatives.[5,6] These compounds have attracted considerable attention as building blocks for polymer synthesis.[5,12-14]

Wastes derived from birch kraft pulp mills and cork industries are produced in large amounts, corresponding to ~3.4 wt %[15,16] and ~23 wt %[17,18] of the total production, respectively. Up to present, their exploitation is often limited to burning in biomass boilers to produce energy. However, substantial valorisation can be attained if valuable components are extracted prior to burning.

Suberin can be isolated from cork and birch outer bark residues by a set of well defined depolymerisation methodologies. They normally require harsh chemical processes of ester bond cleavage through alkaline methanolysis with sodium methoxide, or by alkaline hydrolysis.[5,19] Suberin partial depolymerisation can also be achieved using more gentle (though less efficient) extraction processes, *e.g.* calcium oxide methanolysis.[20-23]

Advances in suberin extraction under milder and environmentally benign conditions will certainly foster its wider application. Recently it has been demonstrated that extraction of suberin from cork can also be attained using cholinium hexanoate as solvent.[24,25] This biocompatible and biodegradable ionic liquid,[26] was able to promote a specific and efficient extraction of suberin from cork. The isolated suberin will certainly display distinct properties from those obtained by conventional depolymerisation methods. This observation, together with the environmental sustainability of the ionic liquid extraction process, opens perspectives for new applications, directly or after chemical modification.

The successful extraction of suberin from cork with cholinium hexanoate[24,25] prompted us to isolate also suberin from birch outer bark. Our aim is to carry out a comparative study focussing on the chemical composition and the thermal behaviour of suberin samples isolated from cork and birch outer bark. The data makes apparent the high versatility of the ionic liquid mild extraction process for the isolation of oligomeric/polymeric suberin fractions displaying a thermal behaviour comparable to that of the starting materials.

3. Materials and Methods

3.1 Cork and Birch outer bark samples

Granulated cork was obtained from the cork producers Amorim & Irmãos SA (Stª Maria de Lamas, Portugal). *Betula pendula* outer bark samples were collected from the debarking line at a birch kraft pulp mill in Finland. The industrial birch outer bark was ground in a laboratory mill to pass a 6-mm screen, followed by separation in water into floating outer bark and sedimented inner bark.

Cork and birch outer bark samples were ground to a powder (< 1 mm) using a centrifuge mill (Retsch) and the soluble extractives removed by Soxhlet extraction with solvents of increasing polarity (dichloromethane, ethanol and water) as previously described by Gil *et al.*[27] The extractive-free powders, hereafter defined solely as cork and birch outer bark (starting materials), were further washed in an excess of deionised water, and then dried prior to use.

3.2 Chemicals

Cholinium hexanoate was synthesised by dropwise addition of hexanoic acid to aqueous cholinium hydrogen carbonate (Sigma ~80 % in water) in equimolar quantities, as described by Petkovic *et al.*[26] The ionic liquid was dried prior to use by stir-heating *in vacuo* (40-50 °C, *ca.* 0.01 mbar). Dimethyl sulfoxide (DMSO) of analytical grade was purchased from Sigma.

3.3 Suberin Extraction

The extraction process followed an optimal methodology previously described by Ferreira *et al*.[25] Briefly, the cholinium hexanoate (T_m = 60.57 °C) was mixed with cork or birch outer bark (ionic liquid : powder ≈ 9:1 wt/wt) and kept at 100 °C during 4 h, with stirring. At the end of the extraction process the mixture was filtrated (DMSO was added, 5 – 10 times the reaction volume, in order to assist the filtration step) and the residue washed thoroughly with an excess of water. The filtrate was kept at 4 °C for a period of

1 h, leading to the precipitation of the extracted suberin, which was then isolated by centrifugation, washed twice with an excess of water, and dried.

3.4 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000 - 600 cm^{-1}, by accumulating 128 scans with a resolution of 4 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v5.0).

3.5 ^{13}C Cross Polarization / Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy (^{13}C CP/MAS NMR)

^{13}C CP/MAS NMR spectra were recorded at 9.4 T on a Brüker 400 spectrometer using 9 kHz spinning rate and MAS with proton 90° pulses of 4 μs. Chemical shifts are given in ppm from glycine. The NMR spectra were processed and analysed with MestreNova v.6.0 (MestreLab Research S.L.).

3.6 Gas Chromatography-Mass Spectrometry (GC-MS)

A Trace GC 2000 Series gas chromatograph equipped with a Thermo Scientific DSQ II mass spectrometer was used. The GC–MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in suberin) relative to *n*-hexadecane (internal standard). Compounds identification was based on the equipment spectral library (Wiley–Nist) and on previously published data, focussing their EI-MS fragmentation patterns and/or retention times.[6,8,19,28] Replicates were done to guarantee low variability and each analysis repeated twice. Each sample was analysed by two complementary methods:

- Method 1, Suberin samples were converted to the corresponding trimethylsilyl (TMS) derivatives and analysed quantitatively by GC–MS, allowing the identification of monomeric structures present in the mixture. In brief, suberin samples (*ca.* 15 mg) were

reacted with 250 μL of pyridine, 250 μL of *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide and 50 μL of trimethylchlorosilane during 30 minutes at 70 °C.[16]

- Method 2, in order to analyse the composition of the oligomeric/polymeric fraction of suberin, samples were, prior to the silylation, submitted to an alkaline hydrolysis to release hydrolysable monomeric constituents. Briefly, suberin samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v), at 95 °C, during 4h.[29] The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1 M HCl, extracted three times with dichloromethane, and the combined organic extracts were dried in a rotary evaporator. Finally, samples were trimethylsilylated as mentioned above, prior to GC-MS analysis.

3.7 Thermogravimetric analysis (TGA)

TGA data were obtained using a TGA – Q50 TA Instruments. All samples were run in crimped aluminium pans with pin-hole under a nitrogen atmosphere (100 cm^3 min^{-1}). Samples were heated up to 600 °C, at a heating rate of 10 °C min^{-1}. Universal Analysis, version 4.4A software was used to determine: degradation temperature ($T_{x\%,\,deg}$), onset temperature (T_{onset}), maximum decomposition temperature ($T_{d,max}$), weight of water adsorbed by the sample in equilibrium with atmosphere (wt_{H_2O}), weight of the solid residue remaining at 600 °C ($wt_{600\,°C}$) and derivative thermograms (DTGA). $T_{x\%,\,deg}$ and T_{onset} were respectively defined as the temperature of a specific weight loss and as the intersection of the baseline weight with the tangent of the weight *vs.* temperature curve as decomposition occurs. $T_{d,max}$ and wt_{H_2O} were respectively defined as the derivative curve (dwt/dT) maximum and the weight loss occurring since the beginning of the experiment until 100 °C.

3.8 Differential Scanning Calorimetry (DSC)

DSC analyses were carried out with a DSC – Q200 TA Instrument. The DSC was calibrated for temperature and heat flow with indium samples and operated under constant purging of nitrogen (50 cm^3 min^{-1}). Samples were hermetically sealed in aluminium pans and heated/cooled up to 120/-80 °C at a constant rate of 5 °C min^{-1},

followed by a 5 min isotherm at 120/-80 °C. Three heating/cooling cycles were repeated. The first cycle was used to clear the sample thermal history. When the second and the third cycles were identical, the latter was used for data collection. The characteristic peaks were analysed using Universal Analysis, version 4.4A software. Melting temperature (T_m) was determined as the maxima of the melting endotherm peak during the heating of the sample.

4. Results and Discussion

Cholinium hexanoate, a biocompatible and biodegradable ionic liquid,[26] was able to promote a highly efficient extraction of suberin from cork, isolated as a partially depolymerised biopolyester.[24,25] The ionic liquid extraction process was in the present study used for the first time to isolate suberin from birch outer bark. A selective extraction of suberin from birch outer bark was attained; similarly to suberin extraction from cork. The extraction and recovery yields were 57.9 and 37.8 wt %; and 48.4 and 39.9 wt % for cork and birch outer bark, respectively. Cork and birch outer bark display a natural abundance of suberin of 30-50 wt % and 40-50 wt %, respectively.[5-10] However, when accounting the removal of extractives, suberin represents typically 50-60 wt % in the starting materials used here. Therefore, it became obvious that while the extraction yield of the process reveals the natural abundance of suberin in the starting material, the recovery yield points out that some losses occurred during suberin precipitation.

DMSO, which does not affect both the efficiency and selectivity of the process, was used to facilitate the filtration step. The process sustainability can be ensured by proper filtration design.[25]

4.1 Chemical Characterisation of suberin

Suberin samples were characterised using ATR-FTIR, ^{13}C CP/MAS NMR and GC-MS analyses. ATR-FTIR spectra of cork and birch outer bark and the corresponding suberin samples extracted by the ionic liquid are depicted in Figure 1. The spectral profiles of both starting materials (cork and birch outer bark) are very similar, as well as those of both suberin samples, and comparable with previously published data for suberised

tissues.[28] Both suberin samples showed the typical suberin features, highlighted in the spectra by vertical lines (Figure 1). Major peaks at 2920 and 2851 cm^{-1} are mainly attributed to the aliphatic chains of suberin, accounting for asymmetric and symmetric C–H stretching vibrations, respectively. The presence of C=O stretching vibration at 1735 cm^{-1} corresponds essentially to the ester groups in suberin. This peak shows also a shoulder at 1712 cm^{-1} that is usually associated with the C=O group of free carboxylic acids; more perceptible in birch outer bark suberin (Figure 1). The aforementioned perfectly agrees with the observation that suberin was partially depolymerised during the ionic liquid extraction process.[25] In addition, the very broad band between 3679 and

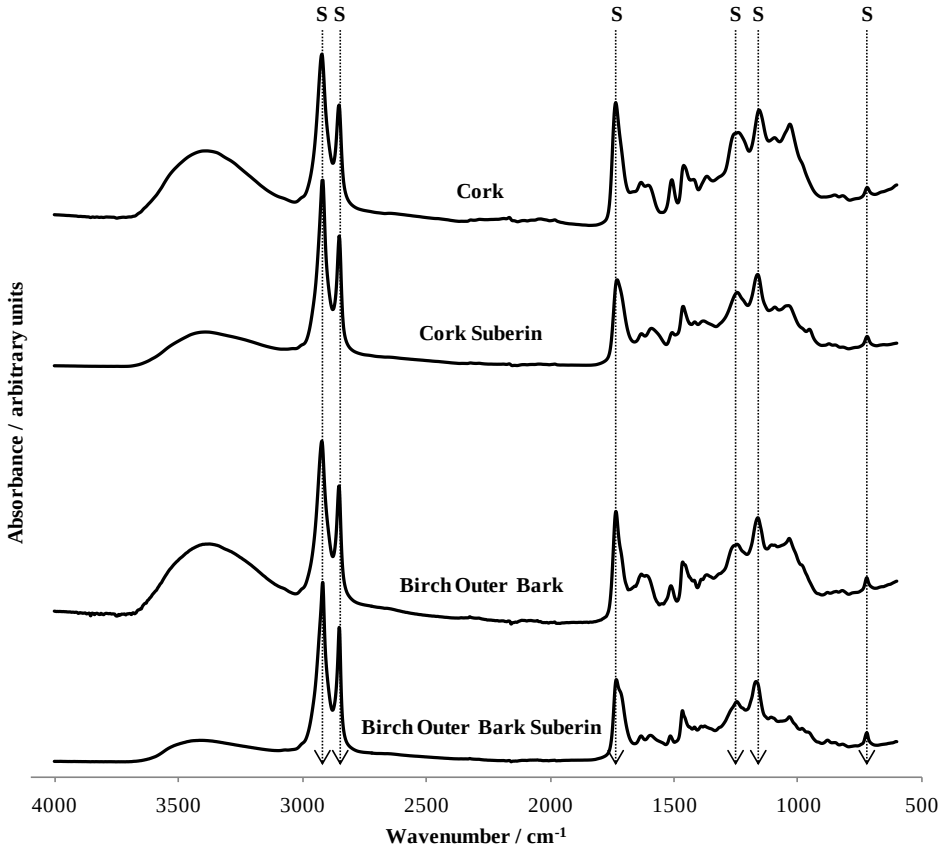

Figure 1| ATR-FTIR spectra of cork and birch outer bark and the corresponding isolated suberin samples following their extraction by cholinium hexanoate. Vertical lines stand for peaks mainly assigned to suberin (S).

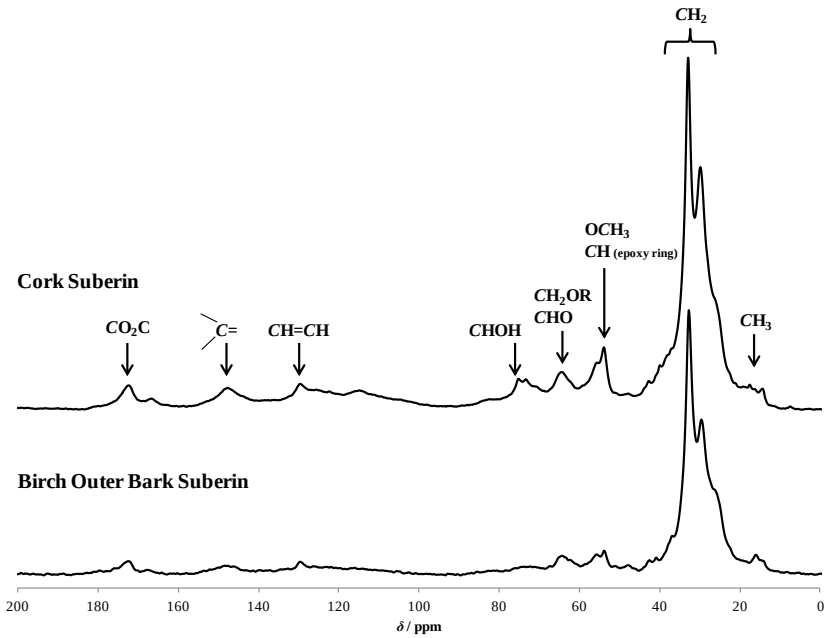

Figure 2| ^{13}C CP/MAS NMR spectra of cork suberin and birch outer bark suberin extracted with cholinium hexanoate. R stands for H or ester group.

3034 cm^{-1} is normally associated with the O–H stretching vibration and can be attributed to carboxylic acids and alcohol groups. Other spectral suberin peaks at 1245, 1164 cm^{-1} and 722 cm^{-1}, corresponding to symmetric and asymmetric C–O stretching and C–H rocking associated with methylenic groups, respectively, were also observed. One cannot disregard the presence of small amounts of lignin (1511, 855 and 819 cm^{-1}) and polysaccharides (1092 and 1034 cm^{-1}).

The ^{13}C CP/MAS NMR spectral information (Figure 2) corroborates the ATR-FTIR data, thus confirming that both extracted suberin samples display fundamentally an aliphatic and esterified nature. The major peaks are assigned in the spectra (Figure 2). Briefly, resonances at δ30/33 ppm and at δ173 ppm are attributed respectively to the methylenic carbons of long aliphatic chains and to carbonyl carbons of ester groups. In both suberin samples the cholinium hexanoate extraction process led to the enrichment of CH_2 groups in the vicinity of oxygen (δ33 ppm), *i.e.* CH_2O groups. A similar effect has been observed in cork suberin extracted using alkaline methanolysis.[8]

The resonances at δ148 ppm are usually assigned to quaternary carbons present in lignin-type structures, which can be associated either to the typical aromatic domains of suberin or lignin.[10,27] Other resonances typical of suberin were also observed, namely δ54, 64, 73 ppm, and δ130 ppm, corresponding to carbons nearby hydroxyl or ester groups and to vinylic carbons, respectively (Figure 2), which can be associated also with the presence of polysaccharides and lignin. The intensities of these peaks, including that at δ148 ppm, were generally higher in the suberin sample isolated from cork relative to that from birch outer bark. Hence it is possible that the natural abundance of lignin and polysaccharides in the starting materials (respectively 25–35 and 15–25 wt % for cork; and 10–15 and 10–15 wt % for birch outer bark)[6,7,9,10] influenced the degree of suberin contamination, if any, with these polymers.

Table 1| Main monomers identified by GC-MS analysis of cork and birch outer bark suberin samples, before and after hydrolysis. Results are given in mg of compound *per* gram of dried starting material.

	Cork Suberin m_x/m_{cork} **mg g^{-1}**		**Birch Outer Bark Suberin** $m_x/m_{birch\ bark}$ **mg g^{-1}**	
Identification (*x*)	**Method 1**	**Method 2**	**Method 1**	**Method 2**
Alkan-1-ols	**0.39**	**2.81**	**0.05**	**0.02**
Octadecanol	0.01	0.04	tr	0.02
Eicosanol	0.03	0.16	—	—
Docosanol	0.24	1.91	0.05	tr
Tetracosanol	0.11	0.70	tr	tr
Alkanoic acids	**2.14**	**4.08**	**0.61**	**8.92**
Hexanoic acid	1.78	0.54	0.24	7.29
Tetradecanoic acid	0.01	0.09	0.02	0.03
Hexadecanoic acid	0.09	0.89	0.13	0.60
Octadeca-9,12-dienoic acid (linoleic acid)	—	0.07	—	0.01
Octadec-9-enoic acid (oleic acid)	0.01	0.08	0.02	0.06
Octadecanoic acid	0.14	1.20	0.06	0.35
Eicosanoic acid	0.01	0.05	0.03	0.06
Docosanoic acid	0.10	1.16	0.11	0.52
Hydroxyacids (hydroxy fatty acids)	**0.66**	**72.30**	**4.33**	**68.20**
10-Hydroxydecanoic acid	0.02	0.15	—	0.17
16-Hydroxyhexadecanoic acid	—	0.65	—	0.31
18-Hydroxyoctadec-9-enoic acid	0.02	12.79	0.16	9.19
18-Hydroxyoctadecanoic acid	—	0.30	0.01	0.21
20-Hydroxyeicos-9-enoic acid	—	0.49	tr	1.49
20-Hydroxyeicosanoic acid	0.01	1.55	0.37	3.82

Table 1| (continued)

	Cork Suberin m_x/m_{cork} mg g^{-1}		Birch Outer Bark Suberin $m_x/m_{birch\ bark}$ mg g^{-1}	
Identification (*x*)	**Method 1**	**Method 2**	**Method 1**	**Method 2**
22-Hydroxydocosanoic acid	0.54	25.81	3.53	16.30
24-Hydroxytetracosanoic acid	0.07	3.00	0.18	0.61
Mid-chain-dihydroxyhexadecanoic acid	—	—	—	2.59
9,18-Dihydroxy-10-methoxyoctadecanoic acid[a]	—	3.35	—	2.41
9,10,18-Trihydroxyoctadecanoic acid	—	20.04	0.08	26.85
cis- *Mid*-chain,18-dihydroxyoctadec-9-enoic acid	—	1.23	—	2.50
trans-*Mid*-chain,18-dihydroxyoctadec-9-enoic acid	—	0.96	—	1.19
Mid-chain-dihydroxyoctadecanoic acid	—	—	—	0.56
Mid-chain, 18-Trihydroxyeicosanoic acid	—	1.98	—	—
α,ω-Diacids	**0.16**	**18.79**	**2.41**	**14.66**
Hexadecanedioic acid	—	0.94	—	0.38
Octadecanedioic acid	tr	0.24	0.08	0.98
Octadec-9-enedioic acid	—	2.39	0.04	4.02
9,10-Dihydroxyoctadecanedioic acid	—	10.02	—	0.93
Eicosanedioic acid	0.08	0.69	0.70	2.34
9,10-Dihydroxyeicosanedioic acid	—	1.18	—	0.16
Docosanedioic acid	0.08	3.33	1.59	5.85
Aromatics	**0.13**	**11.52**	**0.12**	**9.12**
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	tr	0.25	tr	0.06
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	0.08	0.49	0.06	0.12
3,4-Dihydroxybenzoic acid	0.03	—	—	—
4-Hydroxy-3-methoxy-cinnamic acid (*cis*-ferulic acid)	—	0.32	—	0.17
4-Hydroxy-3-methoxy-cinnamic acid (*trans*-ferulic acid)	0.02	10.46	0.05	8.77
Extractives	**7.75**	**16.74**	**13.06**	**28.78**
β-Sitosterol	0.56	0.99	—	—
Friedelin	3.69	3.83	—	—
Betulin	2.63	10.30	12.28	26.70
Betulinic acid	0.87	1.62	0.77	2.08
Monoacylglycerols Derivatives	**3.23**	**0.00**	**1.80**	**0.00**
1-monohexadecanoylglycerol	0.49	—	0.19	—
1-monooctadecanoylglycerol	0.16	—	0.35	—
1-monodocosanoylglycerol	0.44	—	—	—
1-monotetracosanoylglycerol	0.45	—	—	—
1-mono[docosanedi-22-oic acid-1-oyl]glycerol	1.69	—	1.26	—
Glycerol	**0.73**	**0.16**	**0.54**	**0.15**
Others	**1.20**	**9.41**	**2.63**	**4.07**
other epoxy derivatives	—	9.41	—	4.07
n.i.	1.20	—	2.63	—
Total Identified Sample (wt %)	**1.46**	**13.53**	**2.53**	**12.66**

tr – trace amounts; n.i – not identified. [a] Methoxyhidrin artefact from 9,10-Epoxy-18-hydroxyoctadecanoic acid.[28]

The results of the GC-MS analysis of suberin samples are shown in Table 1 and Figure 3. Direct analysis of suberin samples led to very low GC-MS identification yields (Method 1, see section 3.6), namely 1.5 and 2.5 wt % for cork and birch outer bark, respectively (corresponding to 3.9 and 6.3 wt % of the suberin mass). These results suggest that suberin samples extracted by the ionic liquid were still partially polymerized, or at least in the form of esterified oligomeric structures with molecular weights high enough to hamper their detection by GC-MS. These samples showed also to be largely insoluble in most common organic solvents, *e.g.* the dichloromethane insoluble fraction of suberin extracted from cork and birch outer bark represented 42 ± 2 wt % and 30 ± 3 wt %, respectively. These observations suggest that cholinium hexanoate promotes extraction of suberin with still a polymeric/cross-linked nature, *i.e.* likely to be closely related to *in situ* suberin.

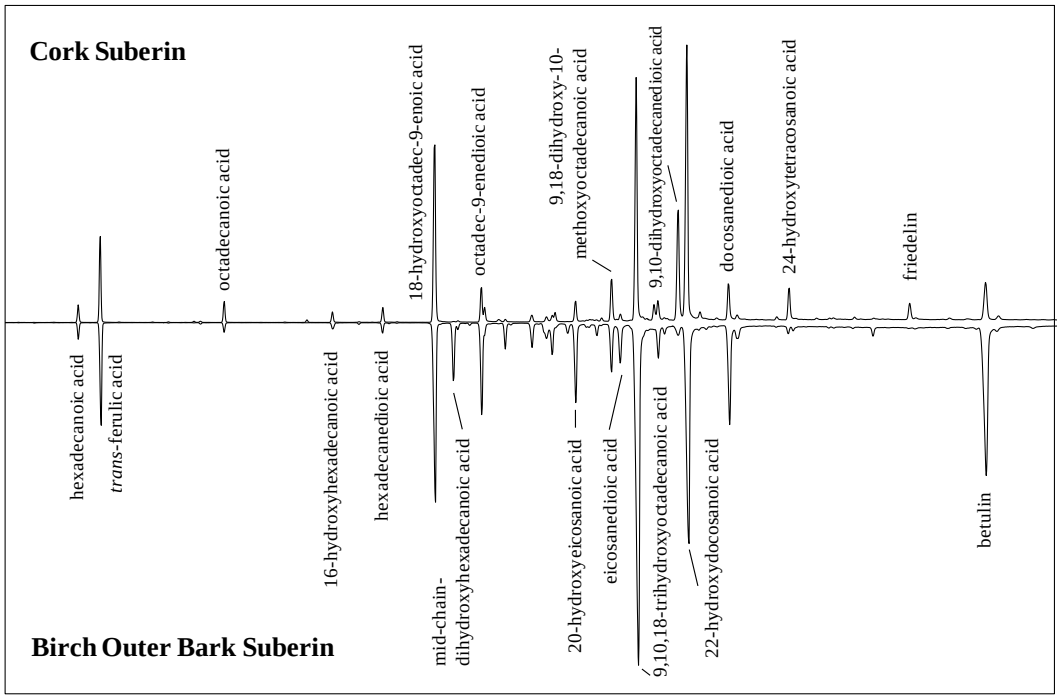

Figure 3| Chromatographic profile of suberin samples extracted from cork and birch outer bark with cholinium hexanoate, as obtained by GC-MS analysis of the hydrolysed samples (Method 2).

In order to circumvent the low GC-MS identification yields, isolated suberin samples were submitted to alkaline hydrolysis (Method 2, see section 3.6) and then analysed by GC-MS (Table 1). The hydrolysed material corresponded to 62.4 wt % and 71.6 wt % of the initial mass of suberin isolated from cork and birch outer bark, respectively. After hydrolysis, the amounts of detected compounds were considerably higher, accounting for approximately 13 wt % of the mass of the starting materials (corresponding to ~35 wt % of suberin mass); in agreement with previously reported results.[5,6,28] The limits in the identification yield are usually attributed to non-volatile high molecular weight fractions.[28]

Both hydrolysed suberin samples reported comparable chemical compositions; as suggested above by the ATR-FTIR and ^{13}C CP/MAS NMR data. These samples showed high abundance of hydroxyacids, α,ω-diacids, extractives, aromatics and alkanoic acids. Other representative compounds, like alkanols and glycerol were also detected in lower amounts. Hydroxyacids detected in cork suberin (72.3 mg g^{-1} of starting material), showed higher abundance of 22-hydroxydocosanoic acid, 9,10,18-trihydroxyoctadecanoic acid followed by 18-hydroxyoctadec-9-enoic acid. On the other hand, in birch outer bark suberin hydroxyacids accounted for 68.2 mg g^{-1} of starting material, mainly composed of 9,10,18-trihydroxyoctadecanoic acid, 22-hydroxydocosanoic acid and 18-hydroxyoctadec-9-enoic acid. Interestingly, both starting materials showed a similar relative abundance of *mid*-chain-hydroxyacids/diacids, some of which might have resulted from ring cleavage of epoxy containing compounds (~40 mg g^{-1} of starting material). This observation differs from previously reported results,[5,6,28] which usually indicate a predominance of epoxy compounds in birch outer bark suberin, especially 9,10-epoxy-18-hydroxyoctadecanoic acid. This dissimilarity can be partially attributed to the selectivity of the extraction process through the use of the ionic liquid. The absence of monomers carrying epoxy rings can also be a consequence of their cleavage, either promoted by the hydrolysis of the suberin samples prior to the GC-MS analyses or by the ionic liquid during suberin extraction. Finally, the relative abundance of aromatics detected by GC-MS in both hydrolysed suberin samples (11.5 and 9.1 mg g^{-1} of cork and birch outer bark, respectively), in particular ferulic acid, reinforces also the findings of ATR-FTIR and ^{13}C CP/MAS NMR analyses.

The GC-MS data confirms that most monomeric compounds detected in suberin, were, prior to hydrolysis, esterified. In addition, these samples also showed some glycerol derivatives, which were not detected in the hydrolysed samples. Glycerol was certainly solubilised in water during the hydrolysis process, as previously reported.[30] In general, the major components detected in the hydrolysed samples are reasonably similar to those regularly detected in suberin samples extracted from cork and birch outer bark by conventional processes.[5,6,8] In addition, most monomeric polyfunctional compounds prone to cross-linking, *i.e.* ≥ 3 OH and/or COOH functionalities, were only detected after hydrolysis of the suberin samples. This strengthens the idea that cholinium hexanoate, regardless of the starting material, extracted suberin owning still a cross-linked nature.

4.2 Thermal Analysis

TGA and DSC analyses were done essentially to evaluate if suberin samples extracted by cholinium hexanoate report a thermal behaviour similar to that of their starting materials.

The TGA thermograms and the most relevant degradation data are shown in Figure 4 and Table 2, respectively. Small weight losses below 100 °C, which are normally associated to water release (wt_{H2O}, Table 2),[31] were detected in all samples. In addition, all the samples were thermally stable up to approximately 200 °C and presented comparable T_{onset}, $T_{5\%wt}$ and $T_{d,max}$ values (Table 2), which highlight the key role of suberin in cork's and birch outer bark's thermal stability. Above 200 °C weight loses were significant and a carbonaceous solid residue was simultaneously formed, which at 600 °C accounted for 10-17 wt % of the initial mass (Table 2).

Table 2| Thermal analyses of cork and birch outer bark and the corresponding suberin samples, namely degradation temperature ($T_{5\%,\ deg}$), onset temperature (T_{onset}), weight of water adsorbed in equilibrium with atmosphere (wt_{H2O}) and weight of the solid residue remaining at 600 °C ($wt_{600\ °C}$).

	Birch Outer Bark Suberin	Birch Outer Bark	Cork Suberin	Cork
$T_{5\%,\ deg}$ / °C	266.8	261.1	232.6	241.5
T_{onset} / °C	369.5	366.3	368.2	348.7
$T_{d,max}$ / °C	422.4	417.1	414.8	407.2
wt_{H2O} / %	0.5	2.1	1.1	2.4
$wt_{600\ °C}$ / %	10.7	15.3	12.9	17.3

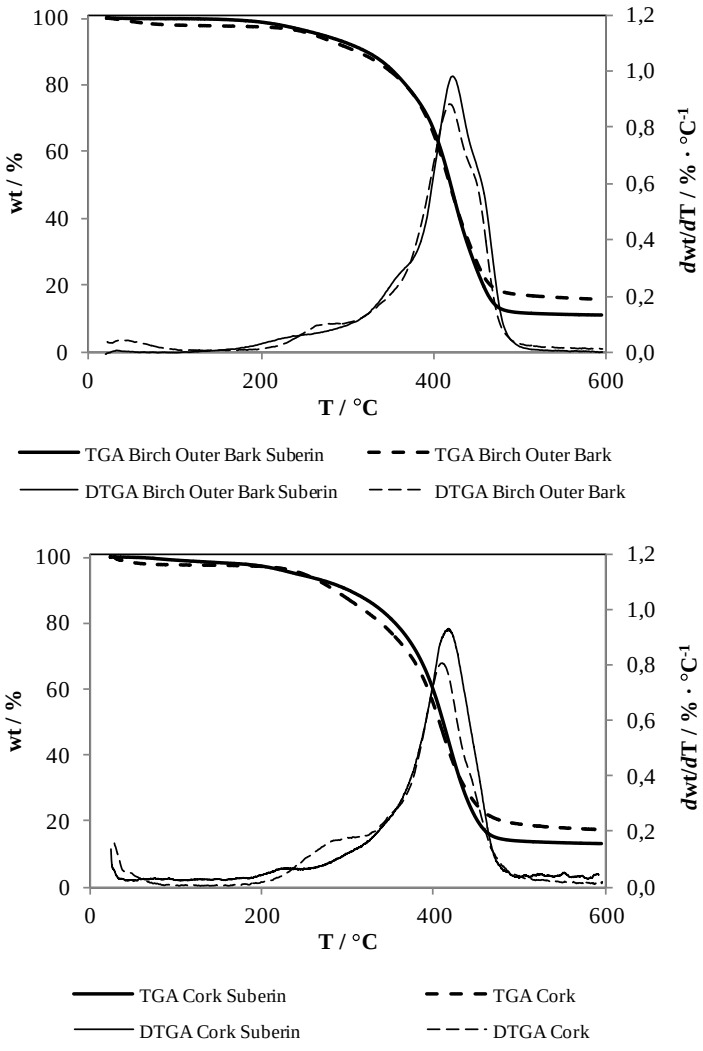

Figure 4| TGA and DTGA curves of cork (bottom) and birch outer bark (top) and the corresponding isolated suberin samples following their extraction by cholinium hexanoate.

The DTGA curves (Figure 4) of the starting materials showed two distinct events during the thermal degradation. The first, a small degradation shoulder occurs at approximately 260-280 °C, which can be mainly attributed to the degradation of hemicelluloses and cellulose.[32,33] This event was more evident in cork due to its higher polysaccharides content. The second event, with a maximum degradation ($T_{d,max}$) in the

range of 400-420 °C corresponds largely to the joint degradation of suberin and lignin.[34,35] Their high thermal stability is due to their *in situ* three dimensional and heavily cross-linked structures.[36]

In general the TGA curves of both suberin samples were similar to those of the starting materials. Nevertheless the shoulder assigned to polysaccharides in DTGA was much less intense. The TGA data put emphasis on the ionic liquid's high selectivity towards suberin. One cannot disregard however that contamination with minor amounts of lignin and polysaccharides might have occurred.

The DSC thermograms (Figure 5) of cork and birch outer bark and of the corresponding isolated suberin samples showed a broad energetic transition that span for several tens of degrees. This correlates well with the multi-component constitution and the low crystallinity of these samples. Cork and birch outer bark presented a broad endothermic peak on heating at 66.6 and 63.1 °C, respectively, while the corresponding suberin samples showed a similar behaviour, with peaks at 71.2 and 50.3 °C. Accordingly, Cordeiro *et al.*[37] associated the melt-crystallisation cycle of suberin with the presence of a microcrystalline phase ($T_m \approx 10$ - 60 °C).

The thermal behaviour of both suberin samples was in general very similar to that of the starting materials.

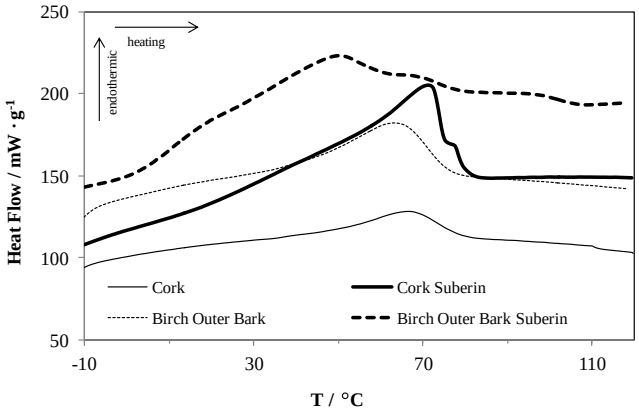

Figure 5| DSC thermograms of cork and birch outer bark and the corresponding isolated suberin samples following their extraction by cholinium hexanoate.

5. Conclusions

The potential of cholinium hexanoate to selectively promote a partial depolymerisation of suberin domains from birch outer bark became evident. This should inspire the application of this extraction process for attempting suberin isolation from other natural sources. Suberin samples extracted from cork and birch outer bark with cholinium hexanoate were observed to be mainly composed of polymeric fractions of suberin-type structures with a comparable thermal behaviour to that of the starting materials. The composing monomeric components were in general similar to those detected in suberin samples obtained by conventional depolymerisation processes. The chemical nature and high thermal stability of the isolated suberin open new perspectives for its use as macromonomers in the development of novel bio-based polymeric materials. Moreover, the extraction of suberin, which is still cross-linked, will certainly provide new opportunities for solving its *in situ* structure, still under debate.

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Chapter IV

Unveiling the dual role of cholinium hexanoate as solvent and catalyst in suberin depolymerisation

The author contributed to the planning and execution of all the experiments described in this chapter, as well as to the data analysis and to the preparation of the manuscript. ^{13}C CP/MAS NMR, GC-MS, HPLC and computational studies were performed by or in collaboration with technicians or co-authors.

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1. Abstract

Disruption of the three-dimensional network of suberin in cork by cholinium hexanoate leads to its efficient and selective isolation. The reaction mechanism, which likely involves selective cleavage of some inter-monomeric bonds in suberin, was still unanswered. To address this question, the role of the ionic liquid during suberin depolymerisation and during cleavage of standard compounds carrying key chemical functionalities was herein investigated. A clear demonstration that the ionic liquid catalyses the hydrolysis of acylglycerol ester bonds was attained herein, both experimentally and computationally (DFT calculations). This behaviour is related to cholinium hexanoate capacity to activate the nucleophilic attack of water. The data showed also that the most favourable reaction is the hydrolysis of acylglycerol ester bonds, with the C2 position reporting the faster kinetics, whilst most of the linear aliphatic esters remained intact. The study emphasises that the ionic liquid plays the dual role of solvent and catalyst and leads to suberin efficient extraction through a mild depolymerisation. It is also one of the few reports of ionic liquids as efficient catalysts in the hydrolysis of esters.

Keywords: Suberin, cholinium hexanoate, ionic liquid, biopolymer, renewable resources, catalyst, hydrolysis, depolymerisation, DFT calculations.

2. Introduction

Renewable resources are increasingly regarded as substituents for highly demanded petroleum-based chemicals.[1] In this context, the extraction, application and biosynthesis of plant biopolyesters, namely cutin and suberin, have been extensively studied.[2-9] Suberin is ubiquitous in higher plants, and particularly abundant in the outer barks of *Quercus suber* and *Betula pendula* and in the peel of *Solanum tuberosum.* This hydrophobic polymeric material is deposited in the secondary cell wall in the internal and the peripheral dermal tissues during cell wall differentiation or as a response to stress and

wounding. Suberin builds an apoplastic barrier that controls the flow of water, gases and ions and protects against biological pathogens and physical aggressions.[9-11]

Since the earliest descriptions, suberin is known to be composed of aromatic and aliphatic monomeric units.[12-14] Currently, the prevailing idea is that suberin comprises two covalently linked domains, the major being the polyaliphatic domain and the minor the polyphenolic one.[10,15-18] Suberin is generally accepted to be organised *in situ* as a lamellar type structure,[16-19] notwithstanding that some structural aspects are still unanswered. The polyphenolic domain is ingrained on the inner face of the primary cell wall.[10,16-18,20,21] Especially due to its high recalcitrancy, the compositional structure of this lignin-like domain is not yet fully understood.[22,23] Even so, it is known to be composed mainly by hydroxycinnamic acids and their derivatives (predominantly ferulic acid) and some vestigial amounts of monolignols (*viz.* p-coumaryl, coniferyl and sinapyl alcohols). Extensive cross-linking between these aromatic monomers in the lignin-like structure, as well as to other cell wall constituents, is done *via* stable carbon-carbon, amide and ether bonds.[9,23-25]

The polyaliphatic domain is composed mostly of long chains (C_{16}–C_{26}) of alkanols, alkanoic acids, ω-hydroxyalkanoic acids and α,ω-alkanedioic acids (and the corresponding *mid*-chain unsaturated, epoxy or *vic*-diol derivatives) and glycerol. In addition, the deposition of hydroxycinnamates leads to the typical lamellar organisation of suberin of alternate aliphatic and phenolic components.[16] These monomers are in a parallel alignment and linked *via* linear aliphatic ester or acylglycerol ester bonds. Glycerol is a key cross-linker in the formation of a three-dimensional network, connecting hydrophilic moieties and both suberin domains.[16,25]

Knowledge on suberin is spread in diverse scientific disciplines, from biology, *e.g.* the understanding of the polymer biosynthesis,[4,7] to chemistry, *e.g.* the depolymerisation and characterisation of suberin[17]. Although only modestly exploited so far, suberin has been regarded as a source of monomers and oligomers for the synthesis of novel macromolecular materials.[9,26-29] Of particular relevance is the abundance of *mid*-chain hydroxy and epoxy fatty acids in suberin. These are rare in other renewable resources and difficult to synthesise chemically.

Suberin extraction from renewable resources is conventionally attained using alkaline hydrolysis[30] or alkaline methanolysis[17,31]. These methods result in extensive ester bond cleavage, *i.e.* depolymerisation. Although with very low extraction efficiency, comparable methods can be used to promote a partial depolymerisation.[17] An alternative extraction method, using cholinium hexanoate, promotes efficient extraction of a still partially cross-linked and highly polymerised suberin, regardless of the renewable resource used, *viz.* cork[32-34], birch outer bark[34] and potato peel (unpublished work). The uniqueness of such method is highlighted by the compositional structure of suberin, hypothesised to be intimately related to that of the *in situ* suberin.[32] Emphasis should be also given to the high biocompatibility and biodegradability of the ionic liquid used in the extraction,[35] which can be recycled and reused throughout the process.[32]

Suberin depolymerisation induced by cholinium hexanoate is likely to involve the selective cleavage of some bonds linking suberin composing monomers. This is the underlying question of the present study: to solve the chemical reaction mechanism behind suberin depolymerisation in cholinium hexanoate media. To address this question, we further investigated the lability of key chemical functionalities of suberin and of standard compounds in the ionic liquid media. The chemical mechanism proposed herein was also supported by DFT calculations.

3. Experimental

3.1 Cork

Granulated cork was obtained from the cork producers Amorim & Irmãos SA (Santa Maria de Lamas, Portugal). The samples were ground to a fine powder (60 mesh) using a centrifuge mill (Retsch) and the cork extractives removed by sequential Soxhlet extraction with solvents of increasing polarity (dichloromethane, ethanol and water) as previously described by Gil *et al.*[36] The extractive-free cork powder, hereinafter defined solely as cork, was further washed with an excess of deionised water for complete removal of low molecular weight compounds, and dried prior to use.

3.2 Chemicals

Cholinium hexanoate was synthesised by dropwise addition of hexanoic acid to aqueous cholinium hydrogen carbonate (Sigma ~80% in water) in equimolar quantities, as previously described.[35] The ionic liquid purity was verified by ^{1}H- and ^{13}C- NMR, CHNS elemental analysis and electrospray ionisation mass spectrometry (ESI-MS). The ionic liquid was dried prior to use by stir-heating in vacuum (40-50 °C, *ca.* 0.01 mbar). The water content, determined by Karl-Fischer titration, was *ca*. 0.2 wt%.

Poly(12-hydroxydodecanedioic acid) was prepared as described before[29] and 9-10-epoxy-18-hydroxyoctadecanoic acid was extracted from *Betula pendula*[37].

Octyl octanoate (≥98%,), glyceryl trioctanoate (≥99%), dimethyl sulfoxide (DMSO, 99.5%), sodium hydroxide (>97%) and dichloromethane (≥99.5%) were purchased from Sigma; glycerol (≥99.5%) from VWR, L-lactic acid (99.5%,) from Fluka and Poly(lactic acid) with an L:D ratio of 96:4 and an M_w of 110000 g mol^{-1} from Cargill-Dow Polymers.

3.3 Suberin extraction

The suberin extraction process followed a methodology which has been previously described.[32,34] Briefly, the cholinium hexanoate (melting temperature, 60.57 °C) was mixed with cork (ionic liquid : cork ≈ 9 : 1 wt/wt) and kept at 100 °C during 1, 2, 4 or 8 hours, with stirring (each in triplicate). At the end of the extraction process, DMSO was added to reduce the viscosity of the mixture,[32] facilitating its filtration through a nylon membrane with an exclusion pore size of 1.0 µm (Millipore, MA, USA). The insoluble residue was then washed thoroughly with an excess of water at 80 °C. Precipitation of the extracted suberin was obtained by keeping the ensuing filtrate (*i.e.* ionic liquid, suberin, DMSO and the water added to wash the insoluble residue) at 4 °C for 1 hour. Suberin was then recovered by centrifugation (30 min at 4 °C and 2450 *g*), washed twice with an excess of water and dried at 50 °C, until constant weight was attained. The aqueous phase (supernatant) was also concentrated in a rotary evaporator and its content in glycerol analysed by high performance liquid chromatography (HPLC).

3.4 Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000-600 cm^{-1}, by accumulating 128 scans with a resolution of 4 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v6.0).

3.5 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H- and ^{13}C-NMR analyses were recorded with a Brüker Avance 400 Ultrashield Plus spectrometer. Spectra were run at 25 °C using standard Brüker pulse programs. ^{13}C CP/MAS NMR spectra were recorded at 9.4 T on a Brüker 400 spectrometer using 9 kHz spinning rate and MAS with proton 90° pulses of 4 µs. Chemical shifts are given in ppm from glycine. The NMR spectra were processed and analysed with MestreNova v6.0 (MestreLab Research S.L.).

3.6 Gas chromatography–mass spectrometry (GC–MS)

A Trace GC 2000 Series gas chromatograph equipped with a Thermo Scientific DSQ II mass spectrometer was used. The GC–MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in suberin) relative to *n*-hexadecane (internal standard). Compounds identification was based on the equipment spectral library (Wiley-Nist) and on previously published data, focussing their EI-MS fragmentation patterns and/or retention times.[30,31,38,39] Replicates were done to guarantee low variability and each analysis repeated twice. Each suberin sample was analysed by two complementary methods:

- Method 1, samples were converted to the corresponding trimethylsilyl (TMS) derivatives and analysed by GC–MS. In brief, suberin samples (*ca.* 15 mg) were reacted with 250 µL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylchlorosilane in 250 µL of pyridine, during 30 min at 70 °C.[40]

- Method 2, samples were submitted to alkaline hydrolysis prior to the silylation, to release hydrolysable monomeric constituents. Briefly, suberin samples were treated

with a solution of 0.5 M NaOH in methanol/water (1:1, v/v) at 95 °C, during 4 hours.[41] The mixture was cooled to room temperature, acidified to pH 3-3.5 with 1 M HCl, and extracted three times by dichloromethane / water partition. The combined organic extracts were dried in a rotary evaporator, then trimethylsilylated as mentioned above, and analysed by GC–MS. The aqueous phases were also concentrated in a rotary evaporator and their content in glycerol analysed by HPLC.

3.7 High Performance Liquid Chromatography

Samples were analysed by HPLC, using a Waters chromatographer, consisting of a 510 Pump, a 715 Autosampler and a Temperature Control Module (Waters Chromatography, Milford, MA, USA), connected to a LKB 2142 Differential Refractometer detector (Bromma, Sweden). Data acquisition was accomplished with the Millenium32 v3.05.01, 1998 system (Waters). Chromatographic separation was undertaken at 60 °C using an Aminex HPX-87H column (300 x 7.8 mm) with 9 μm particle size (Bio-Rad, Hercules, California). Elution was carried out isocratically, at a flow rate of 0.5 $mL \cdot min^{-1}$, with 0.005 N of H_2SO_4 and the sample volume injected was 20 μL. Lactic acid (retention time = 15.98 min) and glycerol (retention time = 15.20 min) quantifications were done using an external calibration curve with high purity standards and within the quantification limits of 0.25 - 20.41 $mg \cdot mL^{-1}$ and 0.25 - 9.99 $mg \cdot mL^{-1}$, respectively.

3.8 Reaction of standard compounds in cholinium hexanoate media

Tests of cholinium hexanoate ability to cleave standard compounds were done in the same conditions used for cork, *i.e.* 100 °C, with stirring (experimental triplicates and technical duplicates).

Glyceryl trioctanoate and octyl octanoate were mixed with cholinium hexanoate at a 1.5 ratio of ionic liquid moles *per* mole of ester bonds, during 1, 2, 4 or 8 hours. At the end of the test, after partition of the ensuing mixture in dichloromethane / water, the organic layer was recovered, concentrated in a rotary evaporator, dried at 50 °C, and then analysed by GC-MS as described in GC-MS - Method 1. The aqueous phases were also concentrated in a rotary evaporator and their content in glycerol analysed by HPLC.

The remaining standard compounds, namely 9,10-epoxy-18-hydroxyoctadecanoic acid, poly(12-hydroxydodecanoic acid) and poly(lactic acid) were mixed with cholinium hexanoate for 4 hours, using a mass ratio (standard: cholinium hexanoate) of 1:4, 1:3, 1:10, 1:10 and 1:10, respectively. For the first two compounds, at the end of the test, an excess of water was added, the precipitate recovered by centrifugation, dried and analysed by ATR-FTIR and/or NMR. In the test with poly(lactic acid), after the reaction, an excess of water was added, the insoluble residue recovered by filtration, dried and then analysed by ATR-FTIR. The content of lactic acid in the aqueous phase was determined by HPLC.

3.9 Thermal microscopy

Thermal microscopy analyses were done using a Leitz Orthoplan polarizing microscope (Wetzlar, Germany) equipped with a JVC digital camera (TK-C130), a Linkham hot stage with a TMS90 temperature controller (±0.5 °C) and a CS196 cooling system.

3.10 Computational Methods

Density functional theory (DFT)[42,43] calculations were performed with the Gaussian 09 software package[44] with the hybrid functional PBE1PBE,[45-48] and the 6-31+G** basis set. Full geometry optimisations including solvent effects (DMSO) were carried out, using the polarisable continuum model (PCM)[49]. The starting structures are based in the most stable approach geometry of the ionic liquid and reactants. Harmonic vibrational frequencies were calculated for all located stationary structures to verify whether they were minima or transition states. Zero-point energies and thermal corrections were taken from unscaled vibrational frequencies. Free energies of activation are given at 25 ºC. All energies were calculated relative to the reagents. All bond lengths are in angstrom (Å) and energies in $kJ \cdot mol^{-1}$.

4. Results and Discussion

The cholinium-hexanoate-based method for suberin extraction combines partial depolymerisation of suberin with high selectivity and extraction efficiency.[32-34] To

identify the chemical bonds prone to be cleaved in the cholinium hexanoate media, we carried out a comprehensive study of the lability of suberin and of standard compounds carrying the key structural bonds in suberin, namely linear aliphatic esters and acylglycerol esters, as depicted schematically in Figure 1.

4.1 Suberin depolymerisation occurs through cleavage of ester bonds: time-course analysis

The natural abundance of suberin in cork is typically *ca.* 50-60 wt%.[10] The extraction of suberin from cork with cholinium hexanoate was extremely fast, given that after one hour the recovery yield was 46.8 wt%. However, after this phase the suberin recovery yield showed a minor increase over time, reaching 54.7 wt% at the eighth hour of extraction (Table 1). Thermal microscopy analysis showed a rapid disruption of cork during the first hour of the extraction. No significant differences were observed for longer periods. Suberin extraction was probably facilitated by this disruption, which exposes the cell wall components to the ionic liquid phase (Supplementary Section S1).

Aiming to identify which chemical bonds in suberin were cleaved in the cholinium hexanoate media, structural composition analyses of the samples recovered

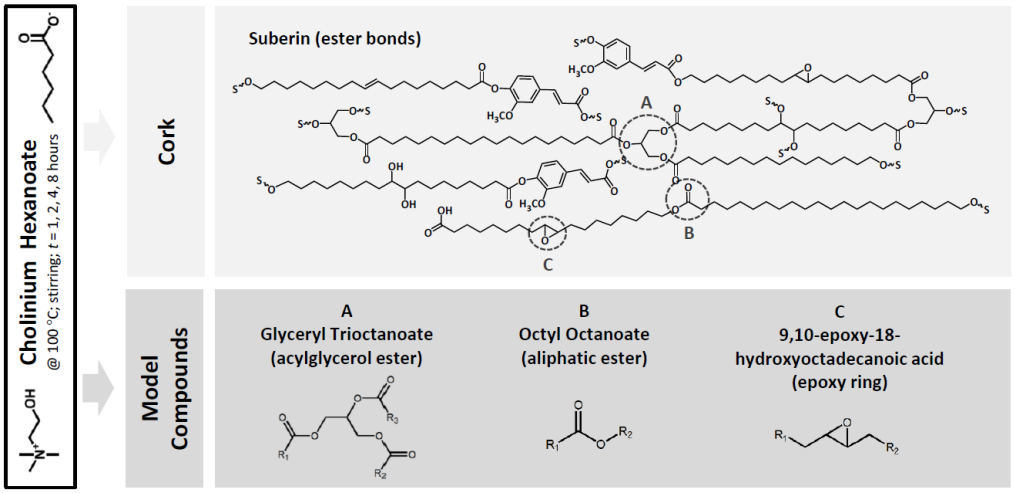


Figure 1| Schematic view of the experiments undertaken to identify the key ester bonds and epoxy groups in suberin prone to be cleaved in the cholinium hexanoate media. Cholinium hexanoate tests with cork (top) and with standard compounds (bottom). The standard compounds reproduce main chemical functionalities present in suberin, namely acylglycerol ester (A), aliphatic ester (B), epoxy ring (C).

along the extraction time were undertaken. After eight hours of extraction, the ester features, namely the resonance at δ 173 ppm (^{13}C CP/MAS NMR, Figure 2A) and the carbonyl stretching at 1731 cm^{-1} (ATR-FTIR, Figure 2B), were still detected in the corresponding spectra. However, the intensity of the ester resonance at δ 173 ppm decreased along the extraction time, suggesting ester bond cleavage. In addition, the peak at 1715 cm^{-1}, which was barely distinguishable in the first hours of the extraction, for longer periods became a defined peak. This peak is associated with hydrogen bonded carbonyl groups in either esters or acids (see magnification in Figure 2B), corroborating the idea that ester bond cleavage occurred. It becomes apparent that the ionic liquid led to mild depolymerisation of suberin through continuous ester bond cleavage.

Table 1| Analysis of suberin extracted from cork by cholinium hexanoate. Values depicted correspond to suberin recovery yield [100·(m recovered suberin / m cork], solubility in dichloromethane [100· (m soluble suberin / m suberin] and alkaline hydrolysis recalcitrancy [100-(100·(m hydrolysable suberin / m suberin] and to the quantification of the glycerol released during suberin depolymerisation, namely in the cholinium hexanoate media (Glycerol[ChHex]) and by alkaline hydrolysis (Glycerol[NaOH]). The second quantifies the hydrolysable glycerol that remained in suberin after the depolymerisation of suberin in the ionic liquid media. Values in brackets stand for standard deviation between replicates.

time / h			**1**	**2**	**4**	**8**
Recovery Yield (STD) / wt%			46.8 (4.5)	47.3 (0.9)	51.0 (5.8)	54.7 (14.8)
Solubility in CH_2Cl_2 (STD) / wt%			46.2 (0.3)	48.0 (1.1)	55.7 (2.9)	65.2 (1.7)
Recalcitrancy to Alkaline Hydrolysis Yield (STD) / wt %			34.4 (3.9)	37.7 (12.4)	49.0 (7.8)	46.3 (0.1)
Glycerol $mg_{glycerol} / g_{suberin}$	**Glycerol$_{[ChHex]}$** [†]	HPLC	17.4 (3.0)	18.6 (3.1)	51.1 (4.1)	63.0 (21.2)
		GC-MS	4.3 (0.4)	5.5 (2.3)	2.8 (1.0)	2.7 (2.5)
		Total	*21.7*	*24.1*	*53.9*	*65.7*
	Glycerol$_{[NaOH]}$ [‡]	HPLC	34.9 (4.5)	32.2 (3.2)	7.6 (1.9)	0.0 (0.0)
		GC-MS	0.8 (0.3)	0.7 (0.2)	0.4 (0.3)	0.3 (0.1)
		Total	*35.7*	*32. 9*	*8.0*	*0.3*
	Total Glycerol		*57.4*	*57.0*	*61.9*	*66.0*

[a] Glycerol$_{[ChHex]}$, glycerol released during suberin depolymerisation in the cholinium hexanoate media, calculated as the total of the glycerol detected by HPLC (*i.e.* solubilised in water during the filtration and the precipitation steps) and by GC-MS (*i.e.* present in the sample).

[b] Glycerol$_{[NaOH]}$, glycerol released during alkaline hydrolysis of suberin samples, calculated as the total of the glycerol detected by HPLC (*i.e.* solubilised in the aqueous phase during dichloromethane / water partition after the alkaline hydrolysis) and by GC-MS (in the sample after alkaline hydrolysis)

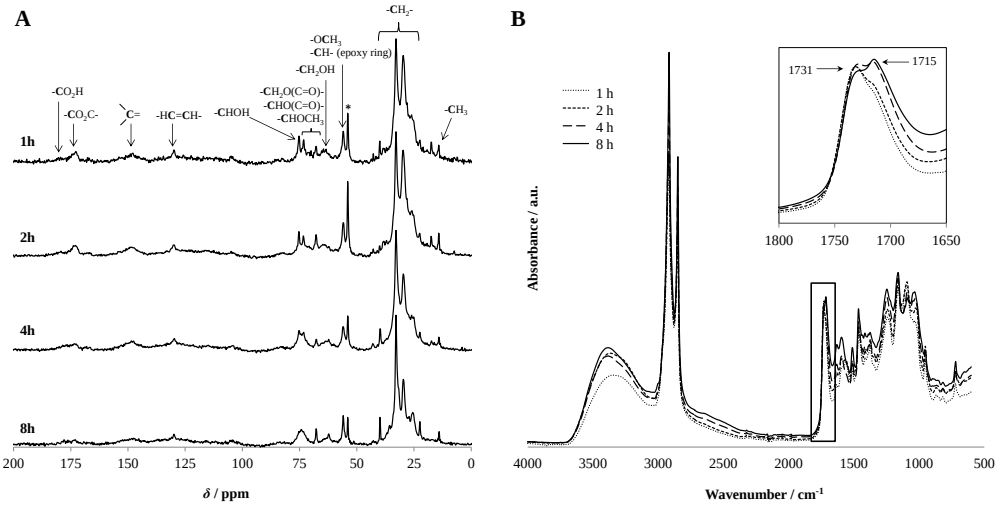


Figure 2| ^{13}C CP/MAS NMR spectra (A) and ATR-FTIR spectra (B) of suberin extracted with cholinium hexanoate after 1, 2, 4 or 8 hours.

Other important observations in the ^{13}C CP/MAS NMR spectra of the recovered suberin samples are the two major resonances at δ 33 and 30 ppm which are associated with aliphatic methylenes (Figure 2A). The ratio 33/30 ppm increased over the extraction time. The carbons at δ 33 ppm have been suggested to report slightly lower mobility in comparison to those at δ 30 ppm[31]. This has been related to the presence of methylene groups near oxygen vicinities (suggestive of linkage to lignin and polysaccharides),[31] or to the presence of crystalline/recalcitrant domains[50,51]. Other resonances typical of suberin can be noticed in all spectra, namely signals at δ 50 - 90 ppm, δ 130 and 148 ppm, assigned to carbons linked to oxygen, vinylic and quaternary carbons, respectively. One cannot disregard the hypothesis that cholinium hexanoate contamination contributed to the resonance at δ 54 ppm (more intense in the samples extracted for one or two hours, see * in Fig. 2A).

The ATR-FTIR spectra of the suberin samples show two major peaks at 2918, 2851 cm^{-1} and a band between 3679-3034 cm^{-1} (Figure 2B). These are respectively associated to the aliphatic chains and to the hydroxyl groups in carboxylic acids and/or alcohols. The hydroxyl band increased along the extraction time. This can be related to the release of hydroxyl moieties, which reinforce the idea that suberin depolymerisation was continuous along the course of the extraction.

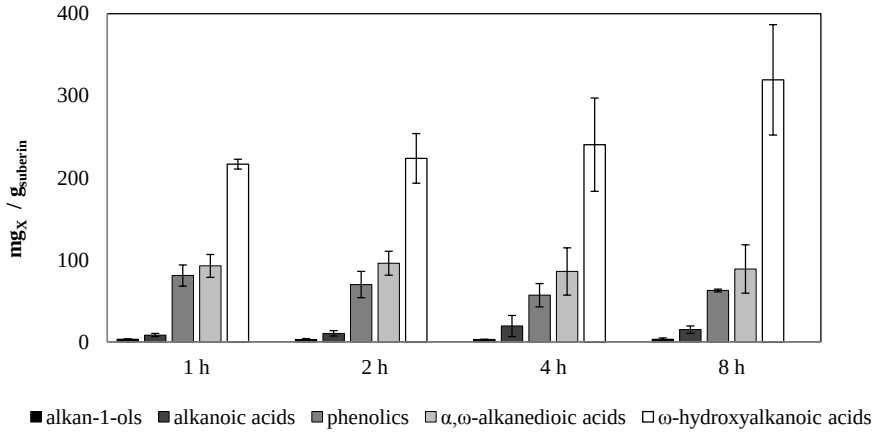


Figure 3| Quantification of suberin monomers in the samples recovered after depolymerisation in cholinium hexanoate media and submitted to alkaline hydrolysis prior to the GC-MS analysis (GC-MS – Method 2). Detailed monomeric quantification in Supplementary Section S2.

More information on the monomeric composition of suberin along the extraction time can be retrieved using GC-MS analysis. The underlying concept here is that suberin extracted with cholinium hexanoate is still cross-linked and/or oligomeric. Consequently, the high molecular weight of these structures hampers their detection by GC-MS (see GC-MS - Method 1, Experimental section). In agreement, very low identification yields were attained (*ca.* 2 - 5 wt%), notwithstanding that the main typical suberin monomers could be detected (data not shown). Monoacylglycerol dimers were only detected in the suberin samples recovered after the first two hours of the extraction, reinforcing the idea that, until the end of the extraction time, oligomers released to the ionic liquid media were further depolymerised.

Aiming to overcome the low GC-MS identification yields, any hydrolysable chemical bonds in the ionic liquid extracted suberin were cleaved by conventional alkaline hydrolysis before the analysis (see GC-MS - Method 2, Experimental section). Consequently, the identification yields of these samples were significantly higher, *ca.* 50 wt% (Supplementary Section S2). In general, the amounts of the monomers herein identified were coherent with those typically reported for suberin extracted with conventional methods.[17,30,31] Major compounds identified were ω-hydroxyalkanoic acids, followed by α,ω-alkanedioic acids and phenolics, and minor ones were alkanoic acids and

alkan-1-ols (Figure 3, Supplementary Section S2). Similar to previous reports,[17] the most abundant monomers herein detected were 22-hydroxydocosanoic acid, followed by ferulic acid, 9,10,18-trihydroxyoctadecanoic acid and 9,10-dihydroxyoctadecanedioic acid.

Glycerol is a major component of suberin,[10,17,52,53] however its high water solubility justifies that the quantities detected by GC-MS, either before or after alkaline hydrolysis of suberin, were very low (Table 1). Most glycerol released from suberin, due to extraction with cholinium hexanoate, becomes solubilised in water during the filtration and the precipitation steps. After alkaline hydrolysis the hydrolysed suberin fraction is recovered by dichloromethane / water partition, consequently most of the hydrolysed glycerol remains in the aqueous phase. In fact, the total glycerol obtained herein is comparable to that reported for conventionally extracted suberin (*ca.* 6 wt%, Table 1).[52,53]

Suberin depolymerisation with cholinium hexanoate released increasing quantities of glycerol, from 21.47 to 67.51 $mg_{glycerol}$ / $g_{suberin}$ ($glycerol_{[ChHex]}$, Table 1). On the contrary, the glycerol released from suberin during alkaline hydrolysis progressively decreases to zero along the extraction time ($glycerol_{[NaOH]}$, Table 1). This means that after eight hours, virtually all hydrolysable acylglycerol ester bonds present in suberin were cleaved in the ionic liquid media. However, significant amounts of suberin monomers were still released after alkaline hydrolysis. Both ester bonds present in suberin, *viz.* linear aliphatic and acylglycerol, are labile to the alkaline hydrolysis. Thus, it seems that the ionic liquid is efficient towards acylglycerol ester bonds, but fails to cleave most of the linear aliphatic esters bonds.

Suberin solubility in dichloromethane and its recalcitrancy to alkaline hydrolysis increased over the extraction time, 46.2 to 65.2 %wt and 34.4 to 46.3 %wt, respectively (Table 1). Upon rapid removal from the cell wall to the ionic liquid media, suberin oligomeric structures undergo continuous depolymerisation - hence solubility in dichloromethane increases. At the same time, non-hydrolysable suberin oligomeric structures are progressively removed from the cell wall - hence recalcitrance increases. All aforementioned data was integrated to propose a model for suberin extraction from cork in cholinium hexanoate media (Figure 4).

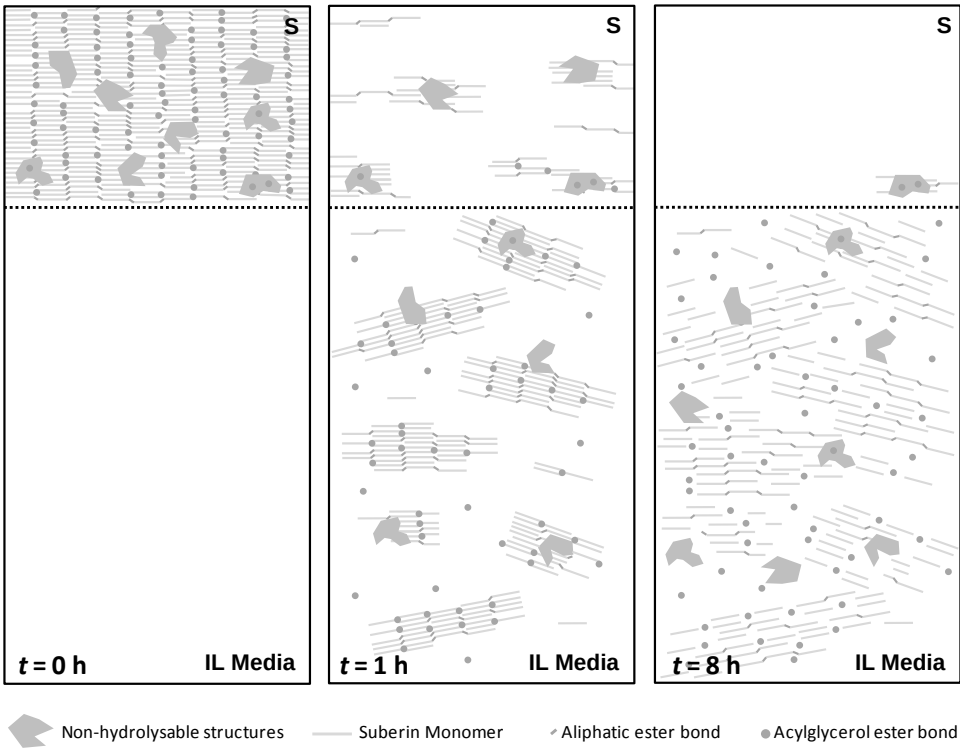


Figure 4| Schematic view of how cholinium hexanoate leads to suberin depolymerisation along the extraction time. S, suberin deposited in the cell wall; IL Media, Ionic liquid media.

4.2 Preferential cleavage of acylglycerol esters in standard compounds

Apparently cholinium hexanoate promoted efficient cleavage of the acylglycerol ester bonds in suberin, but not of the linear aliphatic ester bonds. To further support these findings, the ionic liquid ability to cleave standard compounds carrying linear aliphatic or acylglycerol ester bonds was analysed by monitoring along time the reaction of glyceryl trioctanoate or octyl octanoate, respectively (Figure 1). These standard compounds showed different reaction kinetics (Figure 5). Octyl octanoate was fairly resistant to cleavage. After eight hours of reaction only *ca.* 30 %wt was hydrolysed to octanoic acid and octanol. On the contrary, after the same reaction time, more than 80 %wt of the glyceryl trioctanoate was cleaved, releasing glycerol, octanoic acid and the corresponding diacyl- and monoacyl- glycerols (Figure 5). Based on the low amounts of 2-monoacylglycerol released, it is likely that the most favourable cleavage occurred in the

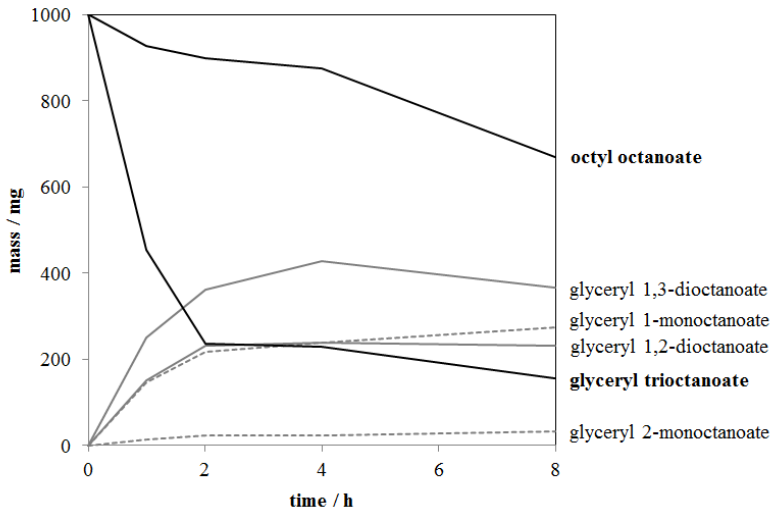


Figure 5| Compounds detected after treatment of glyceryl trioctanoate and octyl octanoate with cholinium hexanoate during 1, 2, 4 and 8 hours. All compounds were identified and quantified by GC-MS. Glycerol, octanol, octanoic acid and error bars were not represented for ease of visualisation (average standard error = 27.8 mg). At time zero, glyceryl trioctanoate and octyl octanoate were assumed to represent the only compounds present in the mixture.

acyl group at position C2 of glycerol. This is suggestive of possible steric hindrance and/or chemical selectivity in the reaction promoted by the ionic liquid. This partially explains why amongst the few monomers identified by GC-MS in the suberin samples extracted with cholinium hexanoate none was a 2-monoacylglycerol.[32,34]

The lack of efficiency of cholinium hexanoate to promote cleavage of linear aliphatic esters was further confirmed using model aliphatic polyesters. After four hours, no alterations were detected in the ATR-FTIR spectra of poly(lactic acid), in particular in the peaks assigned to C=O stretching. Moreover, after this reaction time the mass of the poly(lactic acid) recovered was *ca.* 91.61 ± 0.26wt%, agreeing with the amount of lactic acid detected (6.03 ± 0.63 wt%). The ATR-FTIR spectra of poly(12-hydroxydodecanoic acid) was also unaltered after four hours in the ionic liquid media.

Since the epoxy ring is a significant non-structural functionality in suberin its lability in the ionic liquid media was also preliminary analysed using 9,10-epoxy-18-hydroxyoctadecanoic acid. NMR analysis showed that while the epoxy

ring was partially preserved after 4 hours, some secondary alcohol signals were also detected (^{1}H: 3.5 ppm; ^{13}C: 72 ppm, data not shown). This observation, which was also confirmed by GC-MS and ESI-MS (data not shown), can be associated with the opening of the epoxy ring into a *vic*-diol and/or methoxyhydrin, as suggested above by the ^{13}C CP/MAS NMR data of suberin samples.

Suberin is a structural component of the cork cell wall, thus cannot be removed without impairing its integrity (Supplementary Section S1). The other major polymers in the cork cell wall are likely to be also exposed to the ionic liquid media, namely polysaccharides and lignin, which are linked *via* ether bonds and ether/C–C bonds, respectively. Preliminary data obtained with α-cellulose and alkali lignin, suggested that cholinium hexanoate was unable to promote hydrolysis of their inter-monomeric bonds (data not shown). This further supports cholinium hexanoate selectivity towards suberin.[32-34]

4.3 Cholinium hexanoate as catalyst in the hydrolysis of ester bonds

The cleavage of ester bonds promoted by cholinium hexanoate led to the release of free alcohols and carboxylic acids from glyceryl trioctanoate and octyl octanoate (Figure 5), as well as from suberin (GC-MS Method 1, data not shown). This brings further light to this process: the ester bonds were hydrolysed, implying that the ionic liquid plays the dual role of solvent and catalyst. Water was present in the reaction media, since it was *ca.* 0.2 %wt in cholinium hexanoate and *ca*. 2.4 wt% in cork[34]. Thus the molar fraction of water was 4.9 % relative to the ionic liquid (0.42 wt% in the reaction media and 8.4 wt% relative to suberin).

While the capacity of ionic liquids to play the dual role of solvent and catalyst has been well described,[54] the complex set of chemical and physical interactions, including Coulombic and dipole forces, hydrogen-bonding and acid-base interactions, behind the augmented reactivity in an ionic liquid media are not yet fully understood. Up to now, few hydrolysis reactions in ionic liquid media have been reported, associated to the nucleophilic activation of water.[54,55] It has been demonstrated that, when present at low concentrations, water is dispersed in the ionic liquid as single molecules.[54,55] They form

specific electrostatic interactions and hydrogen-bonding with the ions. As the concentration of water increases, self-association of the water molecules and ion clustering probably occurs, weakening the catalytic effect of the ionic liquid. The chemistry of water in these domains is still a puzzling scientific question.

It was demonstrated that the hydrolysis kinetics is influenced by the ionic liquid hydrogen bonding donor and acceptor ability.[56] Cholinium carboxylates are good hydrogen bond acceptors (Kamlet Taft parameters $\beta > 0.9$)[57]. Accordingly, the mechanism of suberin hydrolysis in cholinium hexanoate media might be related to the nucleophilic activation of water. In fact, increasing the molar fraction of water in the reaction media from 4.9 % to 40%, led to a dramatic reduction in the extraction efficiency of cholinium hexanoate (from 51.0 ± 5.8 to 25.3 ± 2.4 wt%). Water in excess hampered the ionic liquid catalytic effect and/or acted as an anti-solvent. Hence, during suberin depolymerisation from cork, a fine balance of the water concentration in the reaction media needs to be attained.

4.4 Cholinium hexanoate catalyses ester hydrolysis: computational study

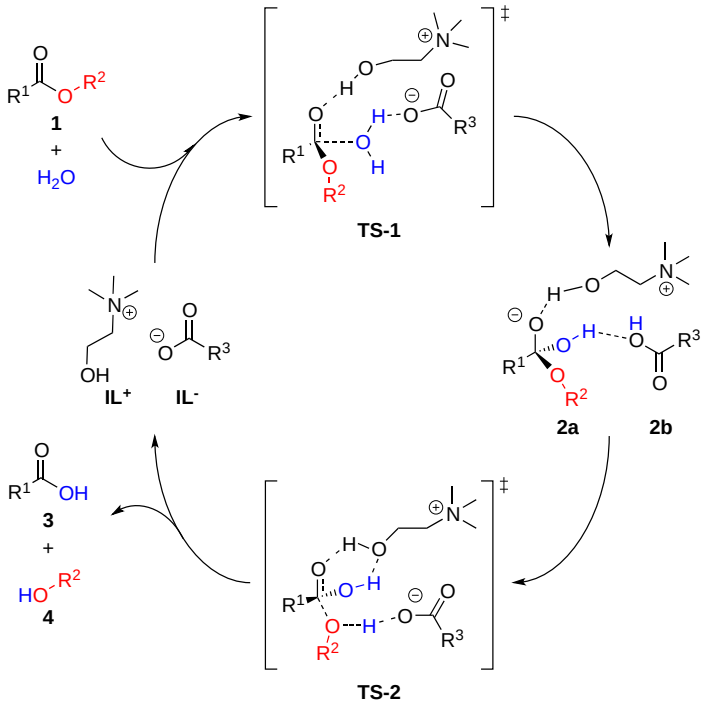
According to the experimental results, cholinium hexanoate catalysed the hydrolysis of esters originating acids and alcohols. A density functional theory (DFT) study[42,43] was performed in order to propose a reaction mechanism. All the intermediates and transitions states (TSs) along the possible reaction pathways were calculated. The proposed reaction mechanism consists of two steps (Scheme 1). In the first step, the hydroxyl group of the cholinium cation (IL^+) establishes one hydrogen bond with the oxygen of the carbonyl group of the ester, favouring the nucleophilic attack of water at the carbon (TS-1). The intermediate **2a** has the newly formed OH bond. In the second step, one proton is transferred from **2b** to the $O(R^2)$ atom, weakening the C-O bond (TS-2). The products, the acid **3** and the alcohol **4**, are formed and the ionic liquid regenerated, closing the catalytic cycle.

The activation barriers obtained for the hydrolysis of four types of linear aliphatic esters (methyl and ethyl acetate, butyl butyrate, and octyl octanoate) catalysed by cholinium acetate or hexanoate are depicted in Table 2 and Figure 6, and show that the

second step of the reaction is the limiting one. The energy difference between TS-1 and TS-2 (Δ (TS-2 – TS-1)) increases with the size of the ester carbon chain, as the linear ester model becomes closer to reality. This arises essentially from the destabilisation of TS-2, since the close proximity of the carbon chain R^1 and R^2 increases the steric repulsion and thus the activation energy. The calculated activation energies are higher in the reaction catalysed by cholinium hexanoate than by cholinium acetate, reflecting the slightly higher pK_a of hexanoic acid (4.88 *vs.* 4.76 of acetic acid). This difference is, however, too small and not significant considering the nature of the model, as the substrates are linear aliphatic esters and the trends may easily be reversed. Since cholinium hexanoate efficiency towards the extraction of suberin was 1.5 fold higher than that of cholinium acetate,[33] this is most probably due to its superior solvent ability towards aliphatic chains. This further emphasises the dual role of the cholinium hexanoate, both as solvent and as catalyst.

The experimental results also showed that the hydrolysis catalysed by cholinium hexanoate was faster in triacylglycerol esters than in monoacylglycerol esters (Figure 5), and that triacylglycerol esters were preferential hydrolysed at the C2 position (Table 3). The reaction mechanism was studied considering the hydrolysis of glyceryl triacetate, catalysed by cholinium acetate, at both positions C1 and C2 (Table 3). The second step (TS-2) is again the limiting one for the reaction at both carbon centres. In addition, the reaction at C2 is faster than at C1 (lower activation energies), in agreement with the experimental results (Figure 5). Thus, as suggested above, it seems that both steric hindrance and chemical reactivity play a role in the hydrolysis of acylglycerol esters in the ionic liquid media.

Finally, the activation energies for the hydrolysis of glyceryl triacetate catalysed by cholinium acetate (Table 3) are lower than those of the linear aliphatic esters (Table 2). This can be correlated to the higher electrophilicity of the ester oxygen atom in the triacylglycerol than in the linear aliphatic ester. These data suggest how cholinium hexanoate might promote a mild depolymerisation of suberin from cork.



Scheme 1| Proposed mechanism for the hydrolysis of esters catalysed by cholinium alkanoates. TS and IL stand for transition state and ionic liquid, respectively.

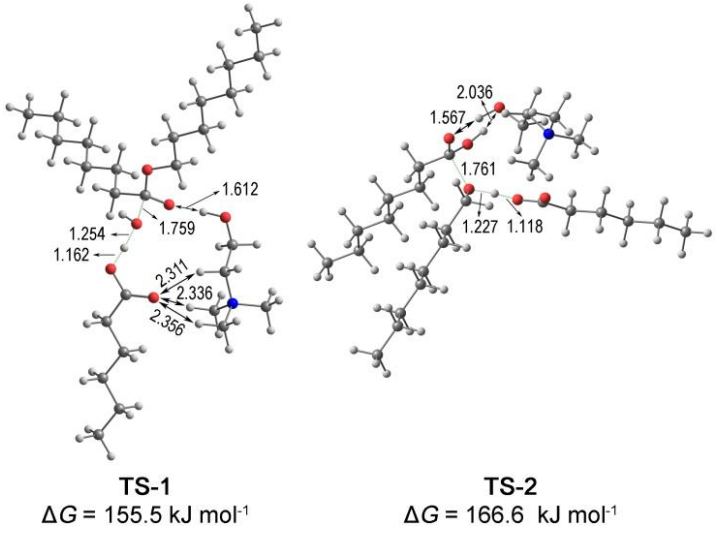
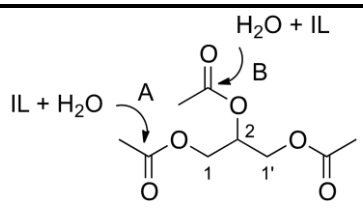


Figure 6| Calculated transition states (TSs) structures for the two steps of the hydrolysis of octyl octanoate catalysed by cholinium hexanoate. All bond lengths are in angstrom (Å).

Table 2| Gibbs activation energies (kJ.mol^{-1}) calculated for the transition states (TSs) presented in Scheme 1.

Ionic liquid	Transition state	Ester			
		methyl acetate	ethyl acetate	butyl butyrate	octyl octanoate
Cholinium acetate	**TS-1**	151.6	153.7	154.8	154.1
	TS-2	151.9	157.4	160.5	163.2
	Δ (**TS-2** – **TS-1**)	0.3	3.7	5.7	9.1
Cholinium hexanoate	**TS-1**	153.6	154.3	156.4	155.5
	TS-2	155.0	161.5	164.2	166.6
	Δ (**TS-2** – **TS-1**)	1.4	7.2	7.8	11.1

Table 3| Gibbs activation energies (kJ.mol^{-1}) calculated for the hydrolysis of glyceryl triacetate catalysed by cholinium acetate.

Transition State		
	Attack A (C1)	**Attack B (C2)**
TS-1	147.8	144.3
TS-2	151.8	146.5
Δ (**TS-2** – **TS-1**)	4.0	2.2

5. Conclusions

Two distinct phases describe the extraction of suberin from cork with cholinium hexanoate. First, rapid removal of nearly whole suberin, still highly polymerised and cross-linked, occurs. This is followed by slow removal of recalcitrant structures, non-hydrolysable and displaying a high cross-link density, concomitantly with continuous depolymerisation of labile oligomers dispersed in the ionic liquid media (Figure 4). The ionic liquid plays the dual role of solvent and catalyst and leads to suberin efficient

extraction through a mild depolymerisation. Disruption of the three-dimensional network of suberin in the ionic liquid media, progresses by preferential cleavage of acylglycerol ester bonds (with the faster kinetics in C2), whilst the linear aliphatic ester bonds remain largely preserved. This reaction mechanism leads to the efficient recovery of a high cross-linked and thus esterified suberin, thus different from that extracted with conventional methods which promote thorough ester cleavage. The ionic liquid catalyses the hydrolysis of esters, hence the water concentration in the reaction media is a decisive parameter. At high concentrations, water weakens the catalytic effect of cholinium hexanoate and/or acts as anti-solvent. A DFT study of the reaction mechanism showed that cholinium hexanoate catalyses the nucleophilic attack of water in two steps. This work should inspire the isolation of biopolyesters from numerous renewable resources, aiming at the development of strategies for their valorisation. A new chemical reaction for the hydrolysis of lipids catalysed by ionic liquids is now available. Ionic liquid chemistry opens the possibility to achieve designer solvents of high performance displaying high selectivity towards a specific carbon centre in an ester bond.

6. Acknowledgements

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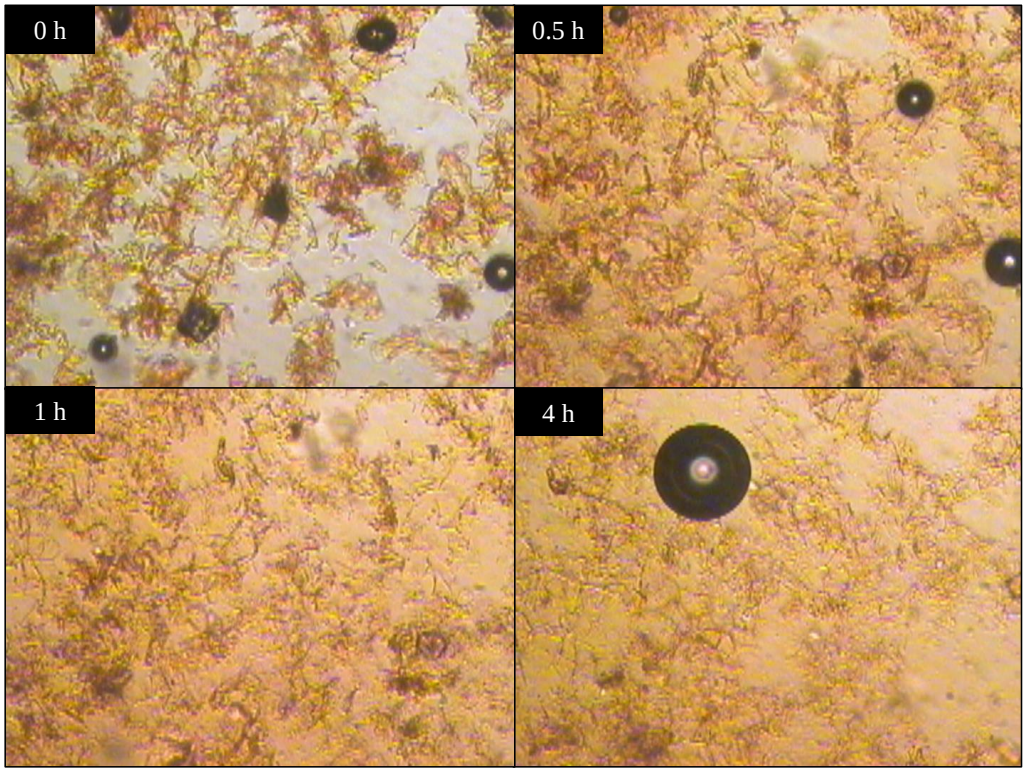
7. Supplementary Information

Section 1 (S1)| Thermal polarised light optical microscopy images of cork during suberin extraction with cholinium hexanoate at discrete times (amplification 20×).



Section 2 (S2)| Main monomers identified by GC-MS analysis of suberin samples after alkaline hydrolysis. Results are given in mg of compound per gram of dried starting material.

Compound, x	$m_x/m_{suberin}$ mg/g (Method 2)			
	1 hours	2 hours	4 hours	8 hours
Alkan-1-ols	***3.26***	***2.60***	***2.88***	***3.32***
tetracosanol	3.26	2.60	2.88	3.32
Alkanoic acids	***8.22***	***10.28***	***19.37***	***14.84***
hexanoic acid[a)]	0.55	0.51	0.16	0.62
hexadecanoic acid	1.07	1.81	8.32	3.34
octadecanoic acid	0.79	1.22	2.25	1.31
octadec-9-enoic acid	0.53	0.59	1.47	0.77
docosanoic acid	4.15	4.00	4.78	7.03
tetracosanoic acid	1.68	2.66	2.55	2.39
ω-Hydroxyalkanoic acids	***216.61***	***223.65***	***240.39***	***319.50***
16-hydroxyhexadecanoic acid	1.91	1.62	1.25	1.86
9,10,18-trihydroxyoctadecanoic acid	56.29	65.41	89.14	101.06
11,12,18-trihydroxyoctadecenoic acid	1.33	1.67	1.19	1.25
18-hydroxyoctadec-9-enoic acid	28.18	28.18	20.53	33.00
9,18-dihydroxy,10-methoxyoctadecanoic acid[b)]	15.60	8.74	0.84	0.00
20-hydroxyeicosanoic acid	5.96	5.72	6.37	6.96
11,12,20-trihydroxyeicosanoic acid	1.75	2.95	4.46	4.18
22-hydroxydocosanoic acid	88.82	89.46	96.67	142.37
24-hydroxytetracosanoic acid	16.77	19.90	19.95	28.83
α,ω-Alkanedioic acids	***92.66***	***95.80***	***85.87***	***88.82***
hexadecanedioic acid	3.42	3.02	1.85	2.08
octadecanedioic acid	1.06	1.08	1.00	0.84
9,10-dihydroxyoctadecanedioic acid	50.29	51.38	49.82	44.32
9-hydroxy,10-methoxyoctadecanedioic acid [b)]	2.41	1.87	0.42	0.63
octadec-9-enedioic acid	8.40	6.99	3.92	4.96
9,10-dihydroxyeixosanedioic acid	1.56	3.23	3.35	2.33
eicosanedioic acid	2.98	2.94	1.56	2.76
docosanedioic acid	18.47	20.43	18.85	27.55
tetracosanedioic acid	4.05	4.86	5.08	3.36
Phenolics	***80.88***	***69.81***	***56.94***	***62.59***
4-hydroxy-3-methoxybenzoic acid (vanillic acid)	7.62	4.35	2.95	3.08
4-hydroxy-3-methoxy-cinnamic acid (*trans*-ferulic acid)	15.71	16.80	11.14	9.89
4-hydroxy-3-methoxy-cinnamic acid (*cis*-ferulic acid)	57.54	48.67	42.85	49.62
Extractives	***23.67***	***36.94***	***24.19***	***19.85***
β-sitosterol	1.40	4.65	9.31	1.45

(continued)

Compound, x	$m_x/m_{suberin}$ mg/g (Method 2)			
	1 hours	**2 hours**	**4 hours**	**8 hours**
friedelin	8.67	10.44	3.58	3.32
betulinol	10.29	14.44	5.93	11.65
betulinic acid	3.31	7.41	5.37	3.42
Glycerol	***0.82***	***0.74***	***0.40***	***0.31***
Unidentified long chain fatty acid derivatives	***64.64***	***53.86***	***5.45***	***14.17***
Identification Yield (wt%)	49.07	49.37	43.55	52.34

a) Hexanoic acid corresponds to the ionic liquid anion, thus it was not accounted for the identification yield.
b) Methoxyhydrin artefacts from the corresponding epoxy acid.

Section 3 (S3)| Cartesian coordinates and computed total energies.

The Cartesian coordinates and computed total energies for the following systems, have been calculated: 1) reactants, 2) reaction between cholinium acetate and methyl acetate, 3) reaction between cholinium hexanoate and methyl acetate, 4) reaction between cholinium acetate and ethyl acetate, 5) reaction between cholinium hexanoate and ethyl acetate, 6) reaction between cholinium acetate and butyl butyrate, 7) reaction between cholinium hexanoate and butyl butyrate, 8) reaction between cholinium acetate and octyl octanoate, 9) reaction between cholinium hexanoate and octyl octanoate and 10) reaction between cholinium acetate and glyceryl triacetate. These data is extremely extensive and are not within the scope of this thesis, thought it can be provided upon request.

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Chapter V

Biomimetic suberin as novel hydrophobic antimicrobial materials

The author contributed to the planning and execution of all the non-biological experiments described in this chapter, as well as to the data analysis and to the preparation of the manuscript. ^{13}C CP/MAS NMR, Zeta potential, XRD, AFM, microscopy, mechanical analysis and SEM were performed by or in collaboration with technicians or co-authors.

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1. Abstract

Biopolymers often have unique properties of considerable interest as basis for new materials. It is however not evident how to extract them from plants without destroying their chemical skeleton and inherent properties. Here we report the *ex situ* reconstitution of suberin as a new hydrophobic and antimicrobial material. In plant cell walls, suberin, a cross-linked network of aromatic and aliphatic monomers, builds up a hydrophobic protective and antimicrobial barrier. Recently we succeeded in extracting suberin from the plant cell wall in the ionic liquid cholinium hexanoate. During extraction the native three-dimensional structure of suberin was partially preserved. In this study, we demonstrate that this preservation is a key factor for *ex situ* spontaneous reconstitution. Without any chemical additives or purification, the suberin composing macromolecules undergo self-association on the casting surface as films. Suberin films obtained show properties similar to the suberin barrier in plant including a potentially broad bactericidal effect.

2. Introduction

Polymers, particularly polyesters, are present in literally every aspect of modern life, from the more mundane to rather sophisticated applications. Up to now, most polyester-based materials still depend on petroleum-based chemistry. Yet polyesters exist in numerous renewable sources and commonly show unique chemical skeletons depending on the source. Differences in skeleton can translate into specific properties, including bio- compatibility and degradability, as well as biological activity. Both suberin and cutin have been in the centre of such efforts[1]. Biopolyesters are widespread in higher plants, deposited in the cell walls as a protective hydrophobic barrier[2,3]. They show high abundance of hydroxyacids and corresponding derivatives carrying *mid*-chain functionalities such as unsaturated, epoxy or *vic*-diol groups, rarely found in other polyester from other sources. They are prominent sources of unique building blocks for development of innovative materials[1]. Biomimetic materials built with hydroxyacids with *mid*-chain functionalities have been produced through polycondensation reactions and the

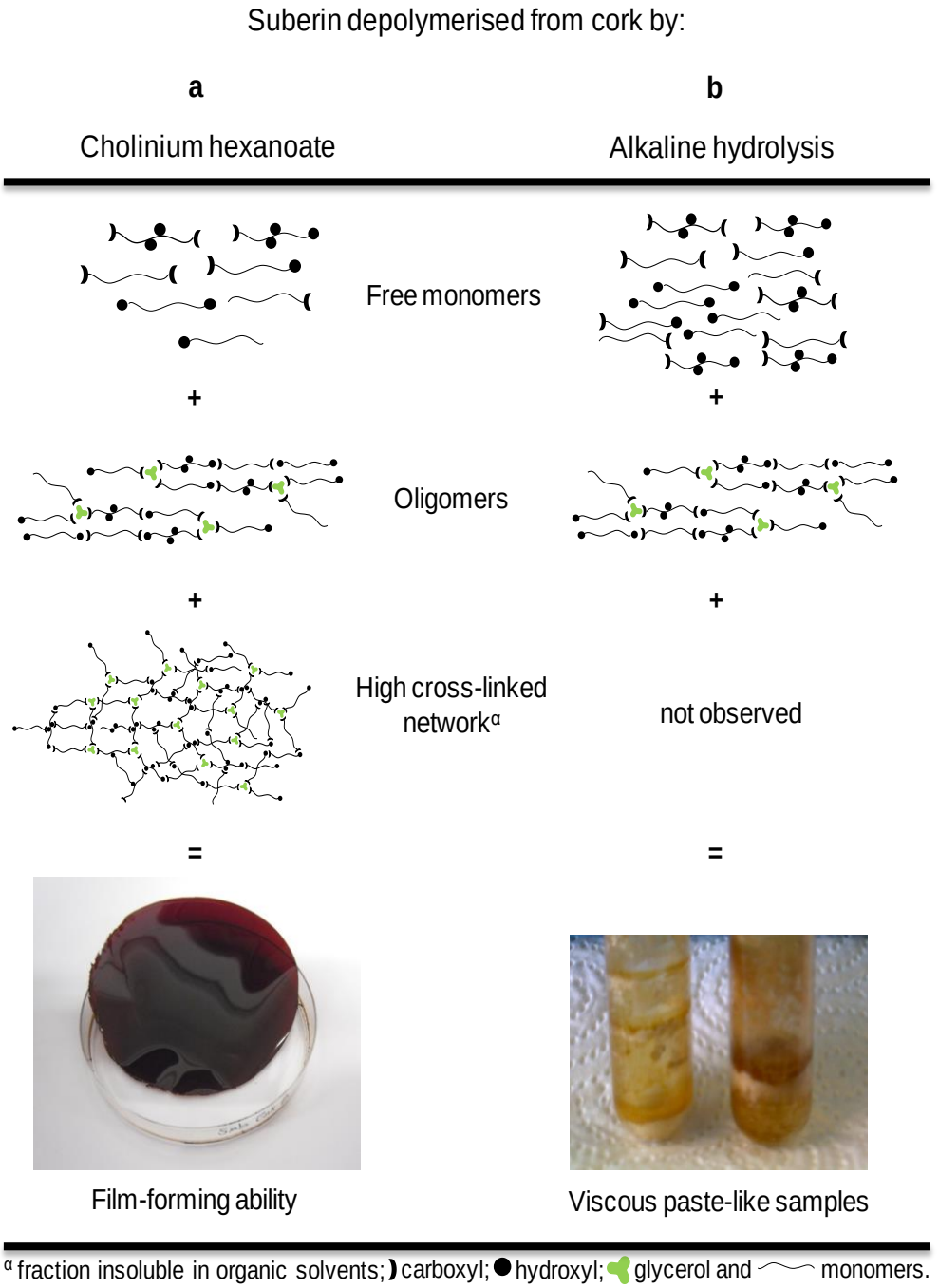

Figure 1| Schematic representation of suberin samples obtained from cork. Suberin depolymerisation through **a**, cholinium hexanoate and **b**, alkaline hydrolysis.

transesterification of either a mix of dissimilar monomers or of a single monomer upon purification[4-7].

Elegant progress has been made but so far the *ex situ* reconstitution of a plant cell wall biopolyester as a material has never been demonstrated. Recent evidence that some cutin purified hydroxyacids undergo self-assembly[8] encouraged us to develop a material mimetic of the biopolyester cell wall barrier, in particular from suberin. Suberin offers strategic advantages over cutin due to its greater diversity of monomers together with its high abundance in *Quercus suber* L. cork (*ca.* 50%wt)[9]. The polyaliphatic domain of suberin also comprises, covalently linked essentially via ester bonds, a minor polyphenolic domain[1,9-11].

Suberin is a structural component of the plant cell wall. At least partial depolymerisation is necessary to efficiently isolate this biopolyester[2,3]. Typically, alkaline hydrolysis or methanolysis that non-specifically cleaves ester bonds have been used, leading to the degradation of the suberin skeleton[1]. More recently we have proposed partial hydrolysis; acylglycerol ester bonds in suberin are efficiently cleaved, whilst linear aliphatic ester bonds are largely preserved[12,13]. This approach uses ionic liquid cholinium hexanoate, in the dual role of solvent and catalyst during suberin extraction from plant cell walls[14]. The macromolecular structure of this extracted suberin, which from now on we refer to as *ex situ* suberin, retains aspects of the native structure. It is built essentially of oligomers of high molecular weight and cross-linked structures plus minor amounts of aromatic and free aliphatic monomers (Figure 1a). The cross-linked structures, which are insoluble in organic solvents, contain non-hydrolysable moieties.

3. Methods

3.1 Suberin film casting process

Suberin samples were extracted as previously described[14]. Briefly, cork was mixed with cholinium hexanoate during 4h at 100 °C, with stirring. The mixture was first filtered to remove solids, the precipitation of suberin in the filtrate was promoted by adding an excess of water (4 °C, overnight), which was then recovered by centrifugation. Suberin

films were produced by depositing a suspension of *ex situ* suberin in water onto a polystyrene plate, followed by slow evaporation of water, first at 30 °C during 4 days and then at 50 °C for 5 days, until complete dryness. Suberin extraction using alkaline hydrolysis[15] and methanolysis with calcium hydroxide[16] was done as previously reported.

3.2 Spectroscopic analysis of suberin films

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). ATR-FTIR spectra were collected on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000-600 cm^{-1}, by gathering 128 scans at a resolution of 4 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v5.0).

X-ray diffraction (XRD). XRD analysis was performed using a Philips X'pert MPD instrument operating with CuK_α radiation (λ = 1.5405980 Å) at 40 kV and 40 mA. Samples were scanned in the 2θ range of 3 to 70°, with a step size of 0.04°, and time per step of 60 s.

3.3 Wettability of suberin films

Water contact angle. Water contact angle measurements (OCA 20, Dataphysics) were performed at room temperature, on both surfaces of the suberin film. A 2 μL drop of distilled water was dispensed on the surface of each film using a microsyringe (Hamilton DS500/GT). Image analysis software (Dataphysics SCA20_M4) was used to calculate the contact angles of the drops by the Laplace-Young method. Six replicates were collected for each suberin surface.

Moisture uptake. Suberin films (dimensions 5 × 45 × 0.1 mm) were conditioned at room humidity of 33, 52 and 91%, achieved by maintaining the samples in closed atmospheric equilibrium at room temperature with saturated solutions of $MgCl_2$, $Mg(NO_3)_2$ and $BaCl_2$, respectively. Three samples were tested for each salt at room humidity. The weight increase due to water absorption was periodically assessed during a period of 21 days.

The water uptake, $wt_{H2O\ uptake}$, at time t was calculated as $wt_{H2O\ uptake} = 100 \times (m_t - m_{t=0})/m_{t=0}$ where m is the mass of the suberin film.

3.4 Mechanical and thermal properties of the suberin films

Thermal properties. Differential Scanning Calorimetry (DSC) analyses were carried out with a DSC – Q200 TA Instrument calibrated for temperature and heat flow with indium samples and operated under constant purging of nitrogen (50 cm^3 min^{-1}). Samples were hermetically sealed in aluminium pans and heated/cooled up to 120/- 80 °C at a constant rate of 5 °C min^{-1}, followed by a 5 min isotherm at 120/-80 °C. Three heating/cooling cycles were repeated. The first cycle was used to clear the sample thermal history. When second and third cycles were identical, the latter was used for data collection. Characteristic peaks were analysed using Universal Analysis, software version 4.4A. Melting temperature (T_m) was determined as the minimum of the melting endothermic peak during the heating cycle.

Mechanical properties. We used a texture analyser (model TA.Hdi, Stable Micro Systems) equipped with a 5 Kg cell and fixed clamps covered with rubber. First mean thickness of the film, an average of five measurements at different points, was calculated using a digital micrometre (model MSC-25S, Mitutoya Crop., Tokyo, Japan, precision 0.001 mm). Film strips (70 mm long, 10 mm wide) were pressed by clamps using an initial grip separation of 50 mm, then their mechanical resistance was tested using a crosshead speed of 0.1 mm/s (eleven replicates). Young's modulus (E), percentage elongation at break (ε_R) and tensile strength or maximum stress (σ_B) were determined from stress-strain curves from uniaxial tensile tests to film failure. All experiments were conducted at room conditions (RT=18°C and RH=33%).

3.5 Antimicrobial assays of suberin films

The antimicrobial dynamic contact tests against the bacteria *Staphylococcus aureus* NCTC8325 and *Escherichia coli* TOP 10 were conducted following the ASTM E2149-01 guideline[17]. Bacterial cultures (50 mL, Müller-Hinton Broth) were incubated in the presence of suberin film pieces (4 mg/mL) and the number of colony forming units

(CFUs) monitored at time points 0h, 2h, 4h, 8h, 12h and 24h by spreading an aliquot of the culture (10 μL) onto solid media (Plate Count Agar). Control cultures (without the suberin film) were also prepared. The results were expressed as follows:

$$\% \text{ kill} = [(CFU_{CONTROL} - CFU_{TEST}) \times 100] / CFU_{CONTROL}$$

To evaluate bacterial viability during exposure to the suberin films (see above), aliquots (1 ml) of the culture were collected at different incubation time points and the cells labelled with propidium iodide and fluorescein diacetate (15 minutes, room temperature, with agitation)[18,19]. Bacterial cells were observed by phase contrast and fluorescence microscopy with a DM5500 B fluorescence microscope (Leica) using a 49 DAPI and N21 filter sets, a 100x magnification objective and images captured with an Andor Luca R EMCCD camera. The micrographs presented here are representative.

3.6 Surface characterisation of suberin films

Microscopy. Phenolic and aliphatic suberin on the surface of the films was detected by microscopy using a 100x magnification objective (see above). Fluorescence microscopy was used to search for aromatic groups on the surface of the films (auto-fluorescent under the UV light) and light microscopy was used to detect the aliphatic groups of suberin upon staining films with Sudan IV (15 minutes, without agitation), as previously described[20]. Micrographs presented here (captured as described above) are representative.

Atomic Force Microscopy (AFM). Tapping mode AFM experiments were performed in a multimode AFM microscope coupled to a Nanoscope IIIa, using a tip Tap300AI-G (BudgetSensors), with a frequency of ca. 300 KHz and a spring constant of 40 N/m, at room temperature and humidity. Scanning speed was optimized to 1.0 Hz and acquisition points were 512 × 512. Height, amplitude, phase and Zsensor were the acquisition channels used with an area of 10 μm, 3 μm and 1 μm (with a resolution of ca. 20nm, 6nm and 2nm, respectively). Imaging data were analysed with the Gwyddion 2.31.

Zeta potential. The zeta potential of suberin films were determined by electrophoretic mobility measured in distilled water by means of a Zeta-Meter 3.0 +.

4. Results and Discussion

Suberin obtained from plant material using conventional extraction methods is a viscous paste-like material devoid of film-forming abilities[1]. Here, we demonstrate that the suberin depolymerised in the ionic liquid media can be reconstituted as a water-proof, hydrophobic and bactericidal film. These properties are similar to the suberin barrier in plant. Upon depolymerisation in cholinium hexanoate media, *ex situ* suberin was recovered simply by promoting its precipitation in water[14] and used without further purification. Suberin films were produced by directly casting a diluted aqueous suspension of *ex situ* suberin on the surface of a polystyrene plate (Figure 1a). Water was removed by evaporation, first at 30 ºC and then at 50 ºC. Production of this biopolyester film requires neither additives nor chemical synthesis. Importantly, the film-forming ability was conserved in *ex situ* suberin samples prepared by freeze-drying of the suspension at -20 ºC, which from now on we refer to as *ex situ* suberin powder. Suberin films present a plastic consistency and are stable, non-leaching after immersion in an aqueous media for at least six months.

The suberin films prepared here prove for the first time that biopolyesters can be reconstituted *ex situ* as a film through the self-association of the composite macromolecules providing the native structure is partially preserved. Given at least partial structural preservation, the composing macromolecules can effectively attract each other, either by hydrogen bonding interactions or hydrophobic interactions. Suberin films and powders had virtually identical ATR-FTIR (Figure 2; see Supplementary Section S3.2 for further details) and ^{13}C CP/MAS NMR spectra (Supplementary Section S1). They are in turn equivalent to those previously reported by us in dried *ex situ* suberin samples[14,21], suggesting no covalent bonds were created between the composing macromolecules during the formation of the film. Additional experiments support the idea that the preservation of the native macromolecular structure ensured film forming abilities of the *ex situ* suberin. Suberin extracted from cork using alkaline hydrolysis which cleaves extensively ester bonds (Figure 2, note peak at 1706 cm^{-1} representative of acid moieties), does not form films (Figure 1b). The last step of this alkaline hydrolysis is

the partitioning of the sample in an organic solvent, discarding the high cross-linked fraction. Even when this step was eliminated to prevent segregation of the sample between the two phases we could not form films. We then used methanolysis with calcium hydroxide, which non-extensively cleaves ester bonds[16]. This partially depolymerised suberin sample (Figure 2) was also viscous and lacking in film-forming ability. Solvent partition of the *ex situ* suberin also obstructed its film-forming ability. With partition in the organic solvent, the insoluble fraction was discarded, *i.e.* high cross-linked structures with approximately 44%wt (Figure 1a). Water plays the role of anti-solvent during the precipitation of the *ex situ* suberin after its depolymerisation in cholinium hexanoate and during casting. This avoids differential segregation of the composing macromolecules, ensuring film forming abilities of the *ex situ* suberin.

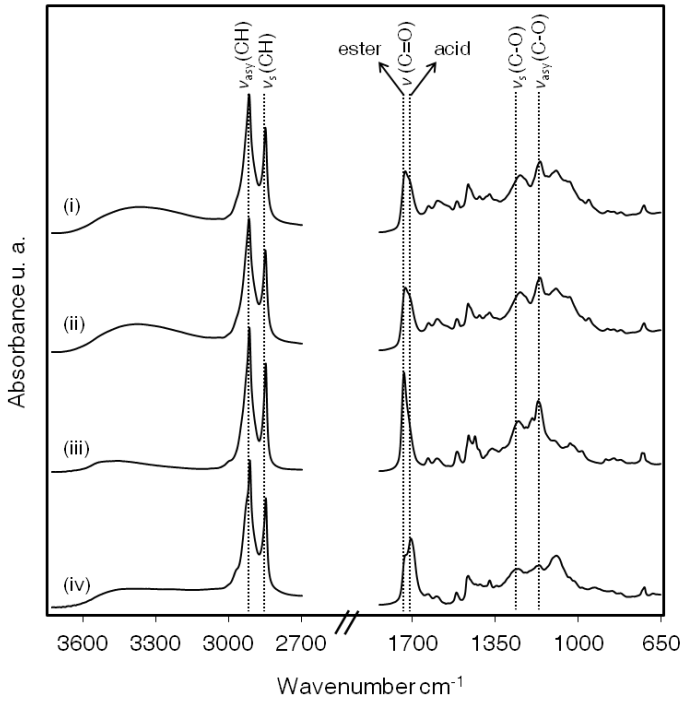

Figure 2| Characterisation of suberin samples by ATR-FTIR spectroscopy. Spectral features of: (i) suberin film produced with *ex situ* suberin (depolymerised with cholinium hexanoate), (ii) suberin powder prepared by freeze-drying of *ex situ* suberin suspensions, (iii) suberin samples prepared by conventional methanolysis with calcium hydroxide and (iv) suberin samples prepared by conventional alkaline hydrolysis with sodium hydroxide.

Glycerol is a key cross-linker in the formation of a three-dimensional network, connecting hydrophilic moieties and both suberin domains[10]. *Ex situ* suberin recovered after 4h of hydrolysis catalysed by the ionic liquid contains only *ca.* 8 mg hydrolysable glycerol *per* g suberin (*i.e.* 10%wt of the total hydrolysable glycerol)[13]. To clarify the role of glycerol we also prepared suberin samples where all the glycerol had already been released (8 h). The 8 h sample showed a lower fraction of cross-linked structures, thus higher solubility in dichloromethane, than the 4 h samples (65%wt and 56%wt, respectively). Most ester bonds different from an acylglycerol ester bond were probably preserved in 4h and 8h samples[13]. Both samples were composed principally of high molecular weight oligomers and cross-linked structures, yet the 8 h sample produced extremely brittle films that were presenting extensive cracks (Supplementary Section S2). Fine tuning of ester bond hydrolysis by the ionic liquid catalyst is therefore vital for film-forming ability of the *ex situ* suberin. This underlines the importance of the mild plus specific depolymerisation, which so far can only be attained using an ionic liquid.

Most *ex situ* suberin is composed of high molecular weight and cross-linked structures, raising the possibility that the reconstitution as a film of this biopolyester is essentially unstructured. We observed that suberin films generated were essentially amorphous. They display the typical X-ray diffraction pattern with a broad amorphous halo (centred *ca.* $2\theta \approx 20^{\circ}$) (Figure 3a). They also showed a broad melting transition,

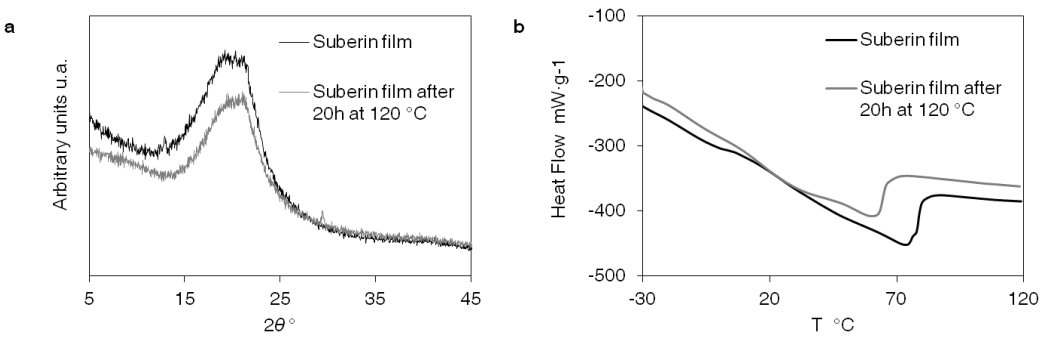

Figure 3| X-ray and thermal characterisation of suberin films. Suberin film and suberin film treated during 20h at 120 °C were characterised by X-ray diffraction pattern (**a**) and DSC analysis (**b**).

spanning from 30 to 70 °C in the DSC thermogram (Figure 3b), indicative of some crystalline domains. The DSC thermogram of the suberin film is virtually identical to that of the *ex situ* suberin powder, reinforcing the idea that the film forms essentially due to self-association of the suberin macromolecules. After 20 h at 120 °C, films were still amorphous (Figure 3a), displayed lower solubility in an organic solvent (1.5 fold reduction) and a more restricted melting transition temperature in the DSC thermogram (Figure 3b). Apparently this treatment induced cross-linking, rather than a higher degree of structural organisation as could be inferred from the DSC data alone. One can hypothesise that hydroxyacids and dicarboxylic acids with unsaturated bonds and epoxy rings existing in the suberin films grant properties similar to other thermosetting biomaterials (*e.g.* castor and soybean oils)[22]. This might prove useful since the films, as predicted, were narrowly elastic and relatively brittle (Young's modulus of 63.2 ± 1.8 MPa, a tensile strength of 1.9 ± 0.1 MPa and an elongation at break 6.7 ± 0.6 %). Films were unable to accommodate substantial deformation as the stress imposed irreversibly affected their three-dimensional network. Cutin materials have been isolated from the fruit cuticles by removing the cell wall polysaccharides through enzymatic digestion[23]. These materials (which are chemically related to suberin films) have been also found to exhibit low elasticity and to be essentially amorphous, whilst preserving some crystalline domains[7,24].

We investigated whether suberin films can act as a water barrier, since suberin in the cell wall forms a barrier which prevents the uncontrolled loss of water and nutrients from plants[3]. They proved to be impermeable to water when using a vacuum filtration device. We then determined the wetting kinetics of the suberin films; both suberin film surfaces can be considered hydrophobic since after 500 ms, usually a sufficient time-scale for a thermodynamic equilibrium to be reached[25], the water contact angle was *ca.* 90°. Suberin films were found to be wettable, albeit with very limited water uptake capacity, 3%wt, 5%wt and 7%wt in a controlled atmosphere of 33%, 52% and 91% humidity, respectively. The spread water on the suberin film surface was rapidly lost when the films were again exposed to low humidity environments. Water drop spreading was also observed; at longer contact periods, the contact angle of water decreased in both surfaces.

After 15 s, it reached *ca.* 65° and was steady up to 90 s. Similar behaviour has been reported for the flat and non-porous surface of an alkaline hydrolysed suberin sample dried over glass or polyethylene[25].

The cell wall suberin barrier is not only virtually impermeable to water and solutes, but is also resistant to microbial hydrolysis and has antimicrobial activity[3,26]. The aromatic moieties confer suberin with antimicrobial features, typical of phenolics. The aliphatic domain physically strengthens the cell wall, protecting it from microbial hydrolysis, and may also exhibit antimicrobial properties[20]. Here we demonstrate that after spontaneous reconstitution of *ex situ* suberin as a film, we preserve the antimicrobial properties of the native suberin. Under dynamic contact conditions suberin films are bactericidal both against Gram- positive *Staphylococcus aureus* and negative *Escherichia coli*. When grown in the presence of the suberin film (4 mg/mL) a dramatic reduction in the number of viable bacteria was rapidly detected. Bactericidal effect (% of kill) was inferred through comparison to the corresponding controls (bacteria grown in broth media without the suberin film). Bacterial death was noticed along the entire growth-curve (Figure 4a), yet more accentuated when the culture reached the stationary phase of growth; after12 h of incubation, growth inhibition was 99.9% and 94.3% for the Gram-positive and the Gram-negative bacteria, respectively (Figure 4a). The number of viable bacteria in the control cultures decreased between the twelfth hour and the twenty-fourth hour. Growth inhibition values reached 98.4% and 94.2% for the two bacterial species (Figure 4a). The bactericidal effect of suberin films was further confirmed by fluorescent microscopy using a LIVE/DEAD assay (Figure 4b-e). The number of dead bacteria largely exceeded that of the live bacteria. Neither tested bacteria was able to degrade the suberin film in any meaningful way; surface composition and morphology were unaltered (Supplementary Section S3). No adhesion of bacteria on the surface of the film was detected, suggesting that the film reduces biofouling.

The anti-biofouling effect might be related to surface topography[27], suggesting that the suberin films might provide a smooth surface. We have used AFM to collect topographic images (tapping mode) of suberin films, both of the superior and inferior surfaces (Figure 4f-k). They show a very low degree of roughness (R_a of

0.024 μm - superior - and 0.002 μm - inferior). They can be classified as smooth or sliding surfaces, which usually are associated with inhibition of cell adhesion. Film surfaces show also a heterogeneous arrangement of "spheroid bumps" (average diameter of 0.41 ± 0.12 μm), some of which are randomly scattered while others overlap. As expected, the film repulsed the AFM tip. Repulsion was slightly more pronounced in regions surrounding the bumps, typical of hydrophobic surfaces. Some bumps had concentrically aligned layers spaced a few nm (*ca.* 5 to 10 nm), remarkably similar to the lamellar organisation of the polyaliphatic domain of suberin of alternate aliphatic and phenolic (*viz.* hydroxycinnamates) components[2,11]. It suggests that some recalcitrant fractions of the suberin lamella where conserved (non-hydrolysable domains), reinforcing the idea that *ex situ* suberin preserved at least partially the native structure.

Long aliphatic cutin monomers have been suggested to undergo self-assembly on hydrophobic casting surfaces following a layered pattern in which molecules align[8]. Despite the higher complexity of suberin the polyaliphatic domain is composed mainly of dicarboxylic and hydroxyacids derived from C_{16} to C_{26}[1,9-11]. It is likely that some long aliphatic chains had self-associated in a similar mode during spontaneous reconstitution of the *ex situ* suberin as a film. Such alignment agrees with the poor electrostatic potential of the film surface as defined by their Zeta potential (ζ = -19.0 mV ± 2.6). The slight negativity of the surface probably derived from the few long aliphatic chains with acidic end groups at the film surface. Long-chain acids and/or alcohols have been postulated to interact strongly with bacterial cell boundaries, leading to their disruption and death[28]. Poor electrostatic potential also agrees with the idea of an anti-biofouling surface[29]. After Sudan IV staining, a dye commonly used to detect lipids and triglycerides[20], most of the surface of the suberin film had a red tinge (Figure 4l), revealing, as predicted, the presence of aliphatic suberin. Despite relatively low levels of hydroxycinnamic acid, its derivatives and monolignols in suberin[11], it has been repeatedly postulated that these compounds are responsible for the antibacterial effect of suberin[20,26]. The association of such polar groups at the surface of the suberin film during the casting in water is also possible. Few phenolic compounds, which auto-fluoresce in the UV

range, were detected at the surface of the film (Figure 4m). It appears that the film's bactericidal effect involves both aliphatic and aromatic compounds.

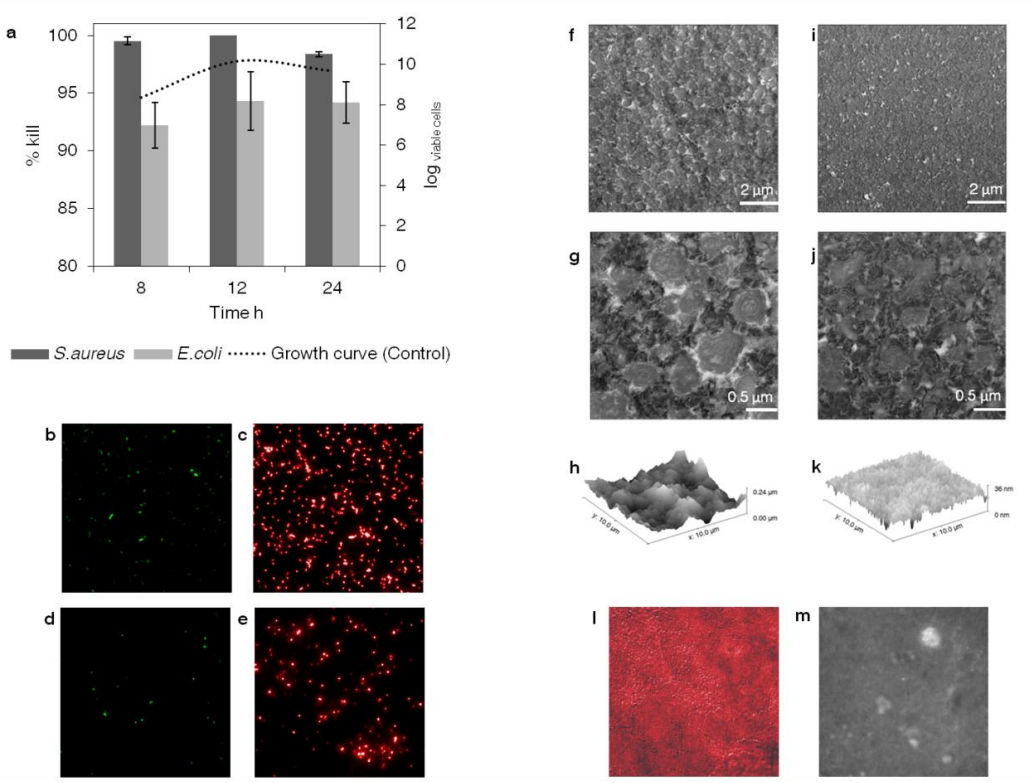

Figure 4| Antimicrobial activity against bacteria and surface characterisation of the suberin film. a, % Kill of *E. coli* and *S. aureus* on suberin films (principal vertical axis) and Log_{10} of viable cell in control cultures (secondary vertical axis). **b-e,** LIVE/DEAD bacterial viability assay of *E. coli* (**b,c**) and *S. aureus* (**d,e**) after 12h. **f-k,** AFM images of the superior (**f-h**) and inferior (**i-k**) surfaces of the suberin films acquired with the channels: amplitude (**f,i**), phase (**g,j**) and height (**h,k**). **l,m,** Histochemical characterisation of the suberin film surface using Sudan IV stain for aliphatic compounds (**l**) and auto-fluorescence of aromatic compounds (**m**).

5. Conclusion

In the present contribution we demonstrated for the first time that plant polyesters can be reconstituted *ex situ* as films, in particular suberin films. We showed that the film forms because the native structure of suberin was partially preserved during its extraction from

the plant cell wall. This was achieved using cholinium hexanoate which acts simultaneously as solvent and mild plus selective catalyst, promoting almost exclusively the cleavage of acylglycerol esters bonds. Whilst glycerol is the key cross-linker in suberin, preservation of linear aliphatic ester bonds uniting different layers of aliphatic suberin secured the partial preservation of the native structure. This, fundamental for the spontaneous reconstitution of the *ex situ* suberin as a film, might explain partially the film's bactericidal properties. We confirmed that suberin films are mimetic of the suberin barrier in plant cell walls. They are hydrophobic and water-proof and show antimicrobial and anti-biofouling properties. This study should inspire development of other biopolyester-based materials for a broad range of applications. One of the first applications we believe will be implemented is clinical usage, also due to the biocompatibility of suberin films.

6. Acknowledgements

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7. Supplementary Information

Section 1 (S1)| NMR spectroscopic characterisation of suberin film and *ex situ* suberin powder

S1.1 – Methods

^{13}C Cross Polarization Magic/Angle Spinning NMR (CP/MAS NMR). The solid state spectra of the *ex situ* suberin powder and the *ex situ* suberin were collected on a Brüker Avance II+ 800 NMR spectrometer operating at 800.33 MHz for proton and 201.24 MHz for carbon, using a TriGama MAS 3.2mm probe with the sample rotating at 12KHz. The *ex situ* suberin samples were finely powdered while mixed with liquid N_2 and then dried, while the powdered *ex situ* suberin was used as-is. A proton 90^o pulse of 5μs, a CP contact time of 1ms and a recycle delay of 2s were used to accumulate 10ms of data points for each sample using 12k scans.

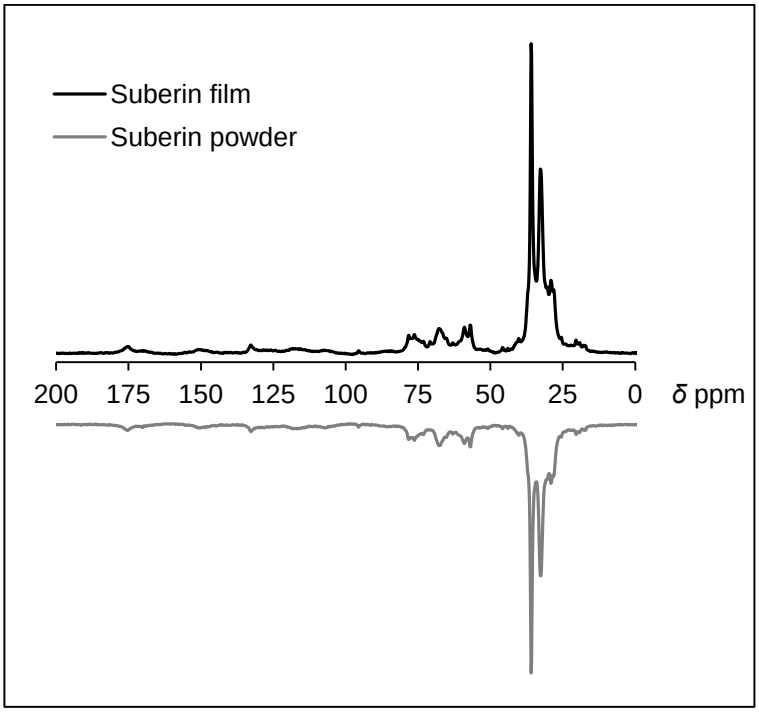

Figure S1| ^{13}C CP/MAS NMR spectra of the suberin film and the *ex situ* suberin powder.

S1.2 - Results

The ^{13}C CP/MAS NMR spectra of the suberin film and the *ex situ* suberin powder showed that both samples own an essential aliphatic and esterified nature. In detail, both suberin samples are characterised by the dominance of aliphatic signals (CH_2: δ 30/33 ppm) and also the presence of ester moieties (C=O: δ 173 ppm and C-O: δ 64 ppm) as a result of the mild depolymerisation promoted by cholinium hexanoate. Other resonances assigned to carbons nearby hydroxyl or ester groups are also present (δ 54, 64, 73 ppm). Typical aromatic signals from suberin were also identified (C=C: δ 130 ppm and 1635 cm^{-1}), although with low intensity. The present data further validate the ATR-FTIR analysis of the suberin film (see Supplementary Section S3.2 for further details).

Section 2 (S2)| Suberin film produced with a suberin sample recovered after 8h of extraction from cork with cholinium hexanoate

S2.1 - Methods

Suberin films cast process was conducted as described in the Main Text. These suberin samples were extracted from cork with cholinium hexanoate during 8h at 100 °C with stirring.

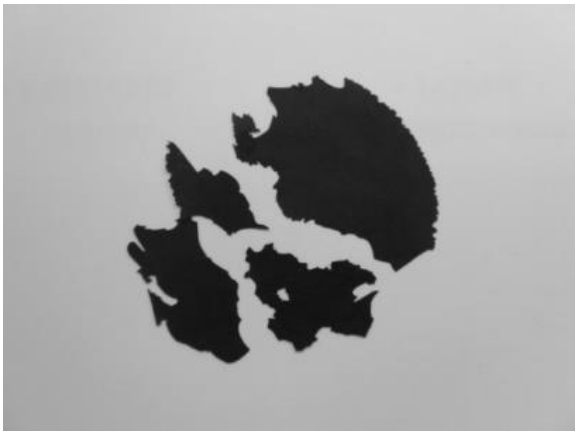

Figure S2| Film prepared with suberin obtained after 8 h of cork depolymerisation with cholinium hexanoate.

Section 3 (S3)| ATR-FTIR spectroscopic analysis and SEM morphologic characterisation of suberin films after incubation with bacteria.

S3.1 - Methods

Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR). The suberin films were collected after incubation with bacteria, dried and analysed by ATR-FTIR as described in the Main Text.

Scanning electron microscopy (SEM). The suberin films were collected after incubation with bacteria. Samples were dried prior to use and coated with a thin layer of gold using a sputter coater (Polaron E-5100). Electron micrographs were recorded using an analytical field emission gunscanning electron microscope (FEG-SEM: JEOL 7001F with Oxford light elements EDS detector) operated at 5–10 kV. The micrographs presented here are representative of the different fractions.

S3.2 - Results

The suberin films were collected after incubation with bacteria, namely *Staphylococcus aureus* (Figure S3ii) or *Escherichia coli* (Figure S3iii), and analysed by ATR-FTIR spectroscopy. Both spectra showed to be identical to the spectrum of an untreated suberin film (Figure S3i). This observation suggests that none of the tested bacteria was able to degrade the suberin films.

All the ATR-FTIR spectra showed to be dominated by two major peaks (ν_{ass} C-H 2920 cm^{-1} and ν_s C-H 2851 cm^{-1}), mostly attributed to the long aliphatic chains of suberin. The polyester nature of the suberin films were further confirmed by the presence of ester moieties (ν C=O 1735 cm^{-1} and ν C-O-C 1164/1245 cm^{-1}). In addition, typical hydroxyl and aromatic signals of suberin are also identified (ν O-H 3679-3034 cm^{-1} and ν C=C 1635 cm^{-1}, respectively).

The morphological surface of the suberin films, after their contact with the selected bacteria, were further analysed by SEM (Figure S4). Again, both suberin films presented no morphological alterations relatively to the untreated suberin film. In addition, no adhesion of bacteria on the surface of the both films was detected. These

observations further reinforce the idea that none of the tested bacteria was able to degrade the suberin film, since its surface composition and morphology were unaltered.

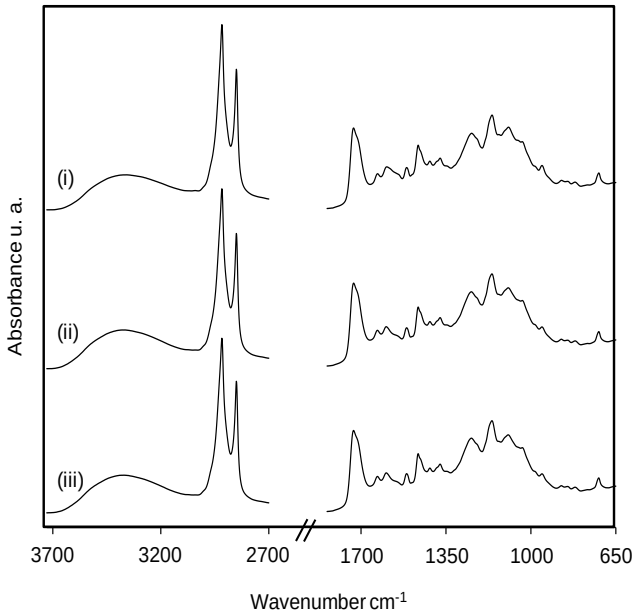

Figure S3| Characterisation of suberin samples by ATR-FTIR spectroscopy. Spectral features of suberin films: (i) untreated, (ii) after incubation with *S. aureus* and (iii) after incubation with *E. coli*.

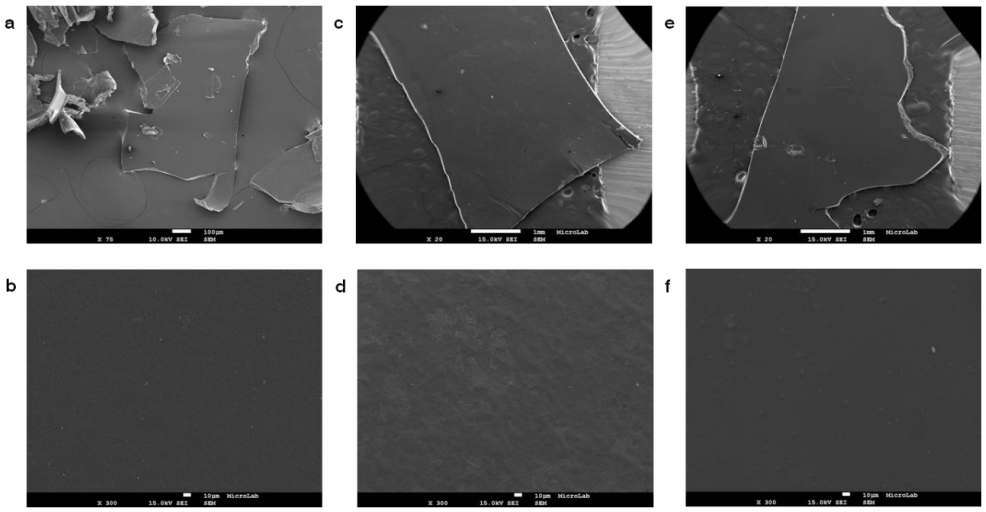

Figure S4| SEM images of suberin films: a,b untreated, **c,d** after incubation with *E. coli* and **e,f** after incubation with *S. aureus*.

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Chapter VI

Microwave assisted extraction of betulin from birch outer bark

The author contributed to the planning and execution of all the experiments described in this chapter, as well as to the data analysis and to the preparation of the manuscript.

Adapted from: R. Ferreira, H. Garcia, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Microwave Assisted Extraction of betulin from birch outer bark, *RSC Adv.*, 2013, in press (DOI: 10.1039/C3RA43868F).

1. Foreword

Given the increasingly interest in betulin, and the successful demonstration of its use as a therapeutic agent, it was considered of major interest the development of an efficient and selective extraction process that could overcome the limitations of the traditional Soxhlet extractions. In recent years, microwave assisted extraction has drawn significant attention, particularly in the isolation of plant metabolites, since high extraction yields can be obtained within short periods and with low solvent consumption.[1, 2] In this section it is demonstrated that the perceived value of birch outer bark residues can be significantly boosted by the synergistic combination of microwave assisted extraction with a selective solvent. For the sake of clarity, in the following lines, microwave assisted extraction basic principles are presented and an overview of the rationale behind this study is given.

Microwaves are electromagnetic radiation, consisting of an electric and a magnetic field oscillating perpendicularly to each other in a frequency that can range from 0.3 to 300 GHz. They can penetrate into certain materials inducing heating by direct effect of the radiation on molecules. Two heating mechanisms are possible, ionic conduction and/or dipole rotation. The first consists in the electrophoretic migration of ions when an electromagnetic field is applied and is exclusive of charged compounds. The second consists in the realignment of molecular dipoles with the electric field of the radiation. The resistance of the surrounding molecules to this forced movements results in molecular friction and collisions where energy is released and dissipated as heat. Therefore, the efficiency of the microwave heating depends on the dissipation factor (tan δ), which accounts for the ability of the solvent to absorb microwave energy and to dissipate it to the surrounding molecules as heat: $\tan \delta = \varepsilon'' / \varepsilon'$. The dielectric loss ($\varepsilon''$) accounts for the conversion efficiency of microwave energy into heat, while the dielectric constant (ε') accounts the polarizability of a molecule in an electric field. Polar molecules

and ionic solutions strongly absorb microwave energy due to the realignment of dipoles and displacement of charged ions, while non-polar solvents are microwave transparent.

Application of ionic liquids in biomass extraction processes has shown promising results, as highlighted in this thesis. Considering the remarkable solvent properties of ionic liquids and their inherent ability to absorb microwave radiation, the use of these solvents in microwave assisted extraction processes seems quite promising.[2] Notwithstanding some studies have reported on the use of ionic liquids for the isolation of terpene-derived compounds,[3-8] few have focused on the use of microwave assisted extraction, and from these only one has considered betulin[5]. Moreover, most of the used formulations consist of imidazolium-based ionic liquids. These are generally regarded as non-benign,[9] and therefore do not meet the requirements established for this work.

Betulin is entrapped, although not covalently bonded, in the peridermal cells of birch outer bark which in turn contains high amounts of suberin. This biopolyester plays a critical role as a structural element in these cells and can be efficiently depolymerised by cholinium hexanoate. Inspired by these observations and by previously reported data,[5] we were motivated to test cholinium-based ionic liquids as a solvent in the microwave assisted extraction of betulin from birch bark. However, as in the study reported by Ressmann *et al.*[5] the performance of the process is hampered by considerable degradation of ionic liquid and by the high levels of contaminants in the crude extracts, namely aliphatic structures arising from depolymerised suberin structures. Comparable levels of contaminants were obtained when performing the extraction in a thermostatic bath. Thus, to achieve acceptable purity levels (> 90%), additional purification steps would be required. These results prompted us to search for alternative solvents highly selective towards betulin. In the scope of this research plan, preference was obviously given to benign solvents. Naturally occurring and commercially available terpenes seemed therefore to be a rational selection since these compounds belong to the same chemical class of betulin. Actually, the careful choice of the microwave solvent revealed to be a critical factor for obtaining pure extracts.

2. Abstract

In this communication, we report a new method for the isolation of betulin, a triterpenoid with interesting pharmacological activities, from birch outer bark. Remarkably high pure betulin raw extracts were obtained, *ca.* 95%, through microwave assisted extraction with limonene. This saves time and energy when compared to the conventional methods.

3. Communication

Plants exhibit an outstanding diversity of complex biologically active molecules, which are difficult, or even impossible, to synthesise chemically. These compounds, which constitute the so called plant extractives, include some which have long been used as therapeutic agents and thus are increasingly inspiring the design of new drugs.[10-13] Plant extractives are normally isolated using simple solvent extraction methods.[1,14,15] However, the compositional variability and the complexity of the obtained extracts together with the poor performance of the extraction method and tight legislation/safety concerns and restrictions, hinder their straightforward application.[12,16] Upon extraction, additional purification steps are usually required to assure satisfactory purity levels. These observations have prompted the development of alternative extraction methods for targeted compounds.[1,14,15]

Betulin (lup-20-(29)-ene-3,28-diol or betulinol, Figure 1) is a naturally occurring lupane triterpenoid particularly abundant in the outer bark of birch species (up to *ca.* 25 wt%).[17-19] Crystalline deposits of betulin rapidly accumulate in peridermal cells during spring and nearly fill the intracellular space in summer.[20,21] The therapeutic and the pharmacological properties of betulin (and its derivatives) have intensively been investigated and properly reviewed in the last decade.[19, 22] In resume, betulin exhibits a wide spectrum of biological activities, such as antitumor, anti-HIV, antibacterial and anti-inflammatory properties. In addition, this triterpenoid can be easily converted in high yields to betulinic acid,[23] a derivative currently in clinical trials[24,25] that shows strong biological activity against HIV and acts as a specific inducer of apoptosis in cancer cells.[19,22]

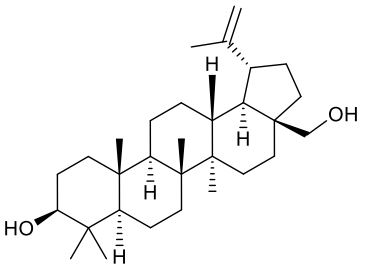

Figure 1| Molecular structure of betulin, the major triterpenoid found in birch outer bark.

Betulin is a non-structural element of the plant cell wall (*i.e.* not covalently bonded), thus it is usually isolated from birch outer bark by conventional Soxhlet extraction using organic solvents such as methanol, ethanol, dichloromethane or chloroform.[17,26-29] Complete removal takes several hours, usually 3 to 8, and requires solvent reflux, meaning time and energy consumptions. The purity level of betulin in these raw extracts is low, typically *ca.* 80 %.[17,19] With the purpose of reaching higher purity levels, alternative extraction techniques and/or solvents have been also tested and include sublimation,[26, 30] supercritical carbon dioxide extraction,[31] ultrasound assisted extraction,[32] ionic liquid extraction[5] and microwave assisted extraction,[5] as well as combination of the aforementioned methods. Even so, betulin extraction is generally followed by one or more purification steps such as crystallisation and/or chromatography.[5,26,27,33]

In recent decades, microwave assisted extraction has drawn significant attention, particularly in medicinal plant research since it allows the isolation of plant extractives in high yields, within a short period and with low energy consumption.[1,2,14,15] Microwaves induce a rapid, efficient and homogeneous heating within the bulk of the extraction medium (solvent and plant matrix), resulting in internal superheating of the water typically present within the plant matrix. This promotes efficient cell disruption facilitating desorption and recovery of the plant extractives. In addition, the presence of molecules with permanent dipoles (*e.g.* water) in the extraction medium also explains why even microwave transparent solvents can be efficiently applied in this technique.[1,14]

In this study, the ability of ethanol and limonene ((*R*)-(+)-limonene) to extract betulin from birch outer bark (milled to < 1 mm) through microwave assisted extraction

was evaluated. Limonene, a commercially available and FDA approved terpene, which belongs to the same chemical class of betulin, seemed to be a rational selection as an alternative solvent. Despite widely used at industrial levels as a flavour/fragrance additive and as degreaser, few studies have reported the use of limonene as solvent, *e.g.* the Soxhlet extraction of terpenoids from plants[34] and the microwave assisted extractions of fatty acids[35]. Notwithstanding that limonene is a moderately microwave absorbing solvent (dielectric constant, $\varepsilon = 2.3$),[35] the presence of low amounts of water in the bark matrix (1.8 ± 0.4 wt%) promotes efficient microwave-heating. The microwave assisted extractions were carried out only during 30 min at isothermal conditions, 150ºC, using a bark to solvent weight ratio of 5 wt%. After this, the bark was removed by filtration (0.45 μm, nylon). When using ethanol the raw extracts were recovered by solvent evaporation, whereas in the case of limonene, it was first precipitated using an anti-solvent, namely cyclohexane or ethyl formate (solvent : anti-solvent volume ratio, 1:2), then recovered simply by filtration (0.45 μm, nylon). The temperature, pressure and power curves of microwave assisted extraction with limonene are depicted in Figure 2. The results obtained using microwave assisted extraction were compared to those obtained by Soxhlet extraction using either ethanol or chloroform as solvents, during 8 hours - a sufficient time period for complete removal of the birch outer bark extractives. After the extraction the samples were recovered by solvent evaporation. The extraction yields and betulin purity levels for all samples were determined by weighing the dried extracts and by GC-MS analysis, respectively (Figure 3).

The extractable fraction of birch outer bark was *ca.* 35 wt%, as determined by Soxhlet extraction using ethanol or chloroform (Figure 3). However, when accounting for the purity of these extracts, the amount of betulin corresponds to *ca.* 25 % of the initial bark weight. This value equals that typically found in birch outer bark.[17-19] Similar extraction yields and purities were obtained after half an hour of microwave assisted extraction with ethanol (Figure 3). This demonstrates that by using microwave assisted extraction, time and energy savings can be promptly attained when compared to the conventional Soxhlet extractions.

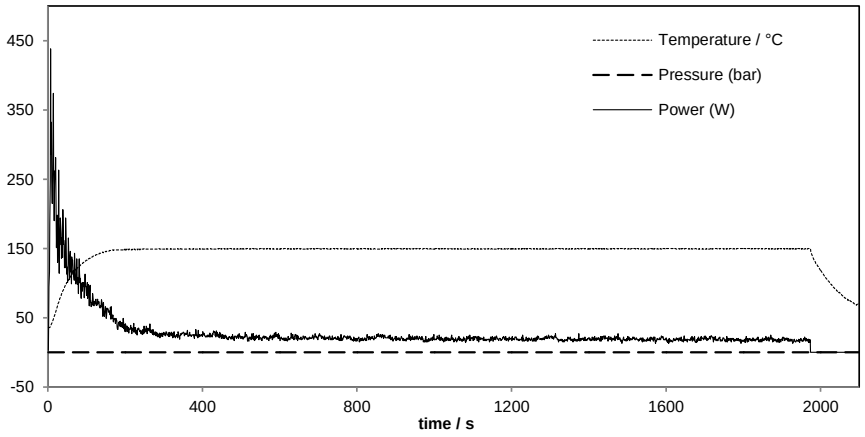

Figure 2| Temperature, pressure and power profiles of a microwave assisted extraction with limonene.

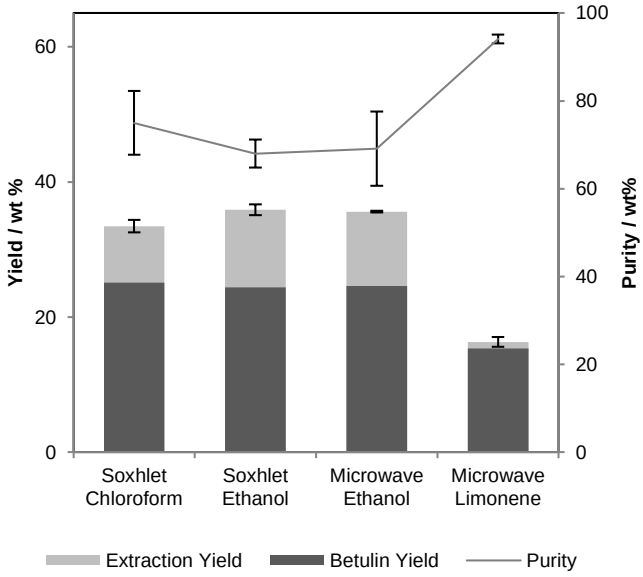

Figure 3| Extraction yields and purity of the betulin raw extracts obtained by soxhlet and microwave assisted extraction. Betulin yields were calculated accounting the extractions yields and their purities.

All the above mentioned betulin raw extracts displayed limited purities, *i.e.* below 75 % (Figure 3). This is due to the low selectivity of the extraction solvents towards the solute. On the contrary, the raw extracts obtained by microwave assisted extraction with limonene showed a remarkably high betulin content, *ca.* 95 wt%. In this case the

precipitated raw extracts accounted for 16.3 % of the initial bark weight (Figure 3). Further improvement of the recovery step is envisaged since limonene was able to dissolve 24.4 % of the initial bark weight. This suggests that limonene dissolved virtually all the available betulin. The high purity of the obtained extracts is highlighted by their clear white colour, whereas the raw betulin extracts obtained either with ethanol or chloroform, regardless of the extraction method, presented a light brown or pale yellow colour, respectively (Figure 4 and Figure 5).

GC-MS analyses confirmed that the raw extracts contain, in addition to betulin, minor amounts of other compounds naturally present in birch outer bark (Table 1, Figure 5).[10,11] Triterpenoid contaminants, in particular lupeol, were predominantly detected in the betulin raw extracts obtained with ethanol and chloroform, whereas almost undetected in those obtained with limonene. Similarly, other compounds such mono- and disaccharides, sterols, long chain alcohols, fatty acids, hydroxycinnamic acids and glycerol were also detected (Table1, Figure 5), although once more almost absent in the extracts obtained with limonene. These observations constitute a further demonstration of the high selectivity of limonene towards betulin, notwithstanding of any minor contribution of the antisolvent to the purity of the extracts.

Table 1| Main components detected in birch outer bark extracts. Chemical identification was done by comparing mass spectra with the equipment mass spectral library (NIST mass spectral library 2005) and their characteristic retention times and fragmentation profiles with published data.

	Amount (Standard deviation) / wt %			
	Microwave Limonene	**Microwave Ethanol**	**Soxhlet Chloroform**	**Soxhlet Ethanol**
Betulin	94,1 (1.0)	69,1 (8.4)	75,0 (7.3)	68,0 (3,2)
Lupeol	0,5 (0.1)	12,3 (1.6)	11,0 (0.5)	9,3 (0,4)
Betulinic acid	3,2 (0.9)	3,1 (0.4)	2,7 (0.3)	2,4 (0,3)
Erythrodiol	0,2 (0.1)	0,4 (0.1)	0,3 (0.004)	0,3 (0,1)
Betulin Aldehyde	0,1 (0.02)	0,8 (0.1)	0,6 (0.03)	0,5 (0,1)
Others[a]	1,9	14,3	10,4	19,5

[a,] includes variable amounts of mono- and disaccharides, sterols, long chain alcohols, fatty acids, hydroxycinnamic acids and glycerol. The amount of these compounds was determined as the difference to 100 wt%.

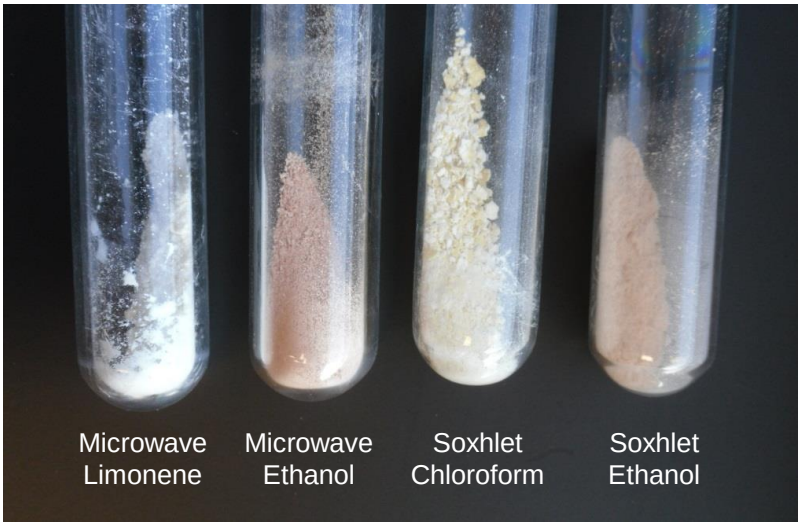

Figure 4| Birch outer bark extracts obtained by Soxhlet and microwave assisted extractions. The extraction method and the used solvent are indicated in the Figure.

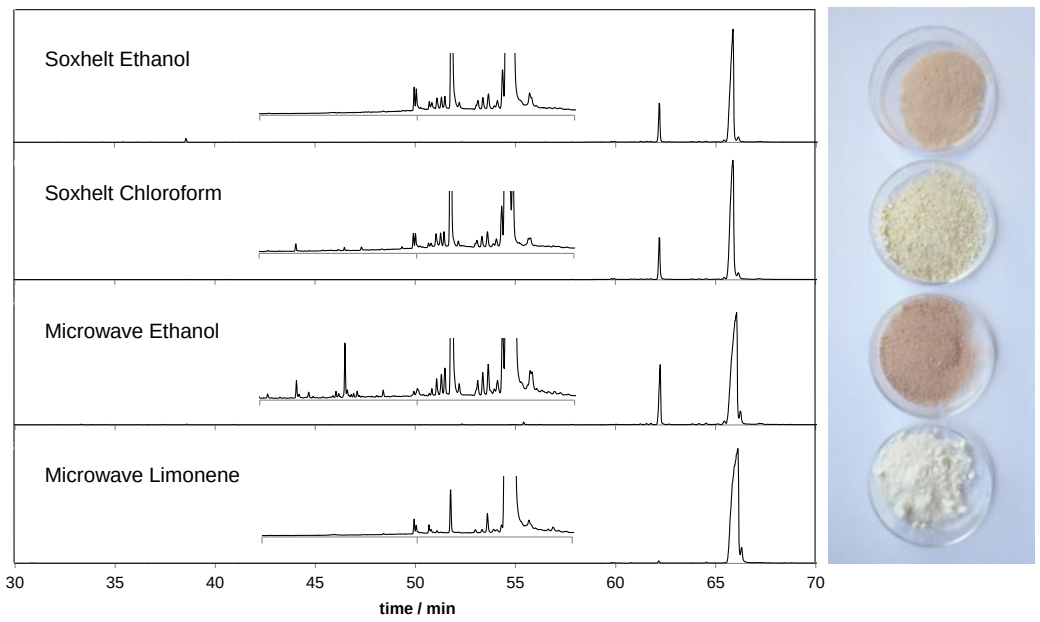

Figure 5| GC-MS chromatograms of the betulin extracts. The amplification shows in more detail the chromatograms between 50 and 70 min. Intensities were normalized in relation to the internal standard peak (n-hexadecane, retention time = 27.4 min).

The betulin raw extracts obtained with limonene through microwave assisted extraction were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and compared to those of standard betulin (98%, Extrasynthese, France). The obtained spectra were virtually similar, supporting the high purity of the raw extracts (Figure 6).

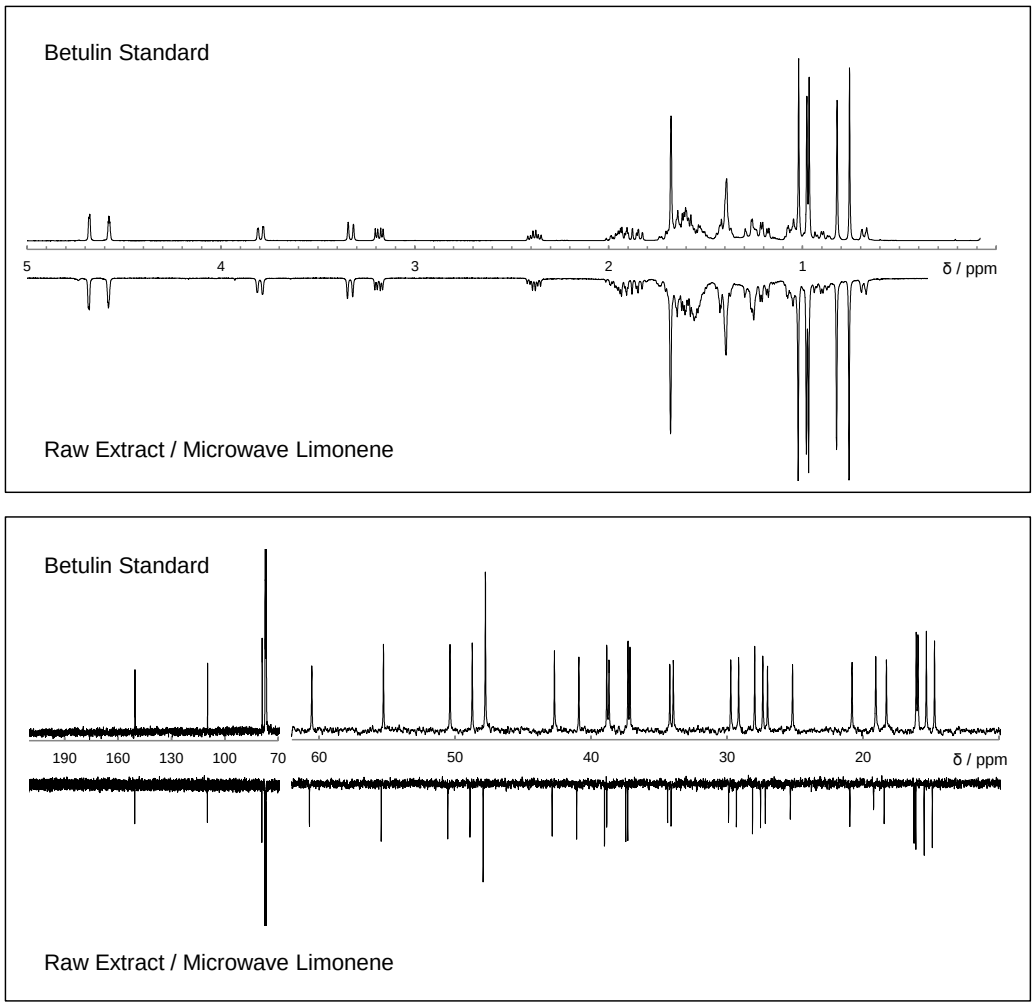

Figure 6| ^{1}H- (top) and ^{13}C-NMR (bottom) spectra of betulin standard compared to those of betulin raw extracts obtained with limonene through microwave assisted extraction.

The data herein presented demonstrate that microwave assisted extraction is a highly efficient technique to rapidly isolate birch bark extractives. In the method herein reported limonene can be recovered simply by evaporation of the anti-solvent used for promoting the precipitation of the extractives. It can be reused, even if containing minor amounts of non-precipitated betulin. To the best of our knowledge, up to date, a single study has focussed on microwave assisted extraction of betulin; however the good performance of the method is hampered by considerable degradation of the solvent.[5]

The findings herein reported constitute an important advance in the isolation of highly pure betulin raw extracts from birch outer bark. It combines the benefits of the microwave assisted extraction technique with the high selectivity of limonene towards betulin. Further work is envisaged, namely the optimisation of the operation conditions through factorial design of experiments. Birch bark constitutes a cheap biomass residue readily available in high quantities, especially in paper pulping industries, where it is burned for energy production.[19] Therefore when considering the limited demand of betulin, birch bark can be regarded as virtually unlimited source of this triterpenoid. For instance, from an average paper mill *ca.* 1800 tons of betulin could be produced annually. These quantities largely exceed the current demands and those of prospective markets.[19] Birch outer bark constitutes also a unique source of suberin,[36] a value-added natural biopolyester that is often regarded as a source of unique building blocks for the development of innovative materials. The integrated extraction of suberin and betulin from birch bark would certainly boost the value of this residue. Notably, the developed knowledge should encourage the application of this process to the selective extraction of other triterpenoids from numerous biomass residues.

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Chapter VII

Discussion: Impact and Perspectives

The research described in this thesis aimed at the development of alternative methods for the up-grading of cork and birch outer bark residues. The following lines provide a critical evaluation of the work herein described highlighting its scientific impact, possible improvements and the "greenness" of the processes.

1. Suberin mild depolymerisation in cholinium hexanoate

The unique properties and the physiological role of suberin in cork and birch outer bark have been increasingly inspiring the design of new biomimetic materials.[1, 2] It is however not evident how to extract this biopolyester from suberised cell walls without impairing its structure and inherent properties. Conventional processes to extract suberin from cork either result in *i)* extensive depolymerisation and complete extraction or *ii)* mild depolymerisation and low extraction yields.[1] The present work described for the first time a mild depolymerisation process that allows the complete and selective removal of suberin, partially preserving its structure. Noteworthy, such pioneering process was attained with cholinium hexanoate, constituting also the first demonstration of plant biomass dissolution with biocompatible and biodegradable ionic liquids. In fact, the study of suberin extraction mechanism revealed that cholinium hexanoate is more than a simple solvent. It also acts as a catalyst towards the selective hydrolysis of acyl-glycerol esters, while the linear aliphatic ester bonds are largely preserved. Importantly, the partial preservation of suberin cross-linked and polymeric nature rendered possible the production of suberin films for the first time. These are produced simply by casting a suberin aqueous suspension without any chemical additives or purification steps. Interestingly, the film forming ability of this suspension is *per se* rather uncommon and probably reveals the ability of suberin macromonomers to self-associate. As a result of the partial structure preservation, suberin films exhibit a set of properties that resemble its physiological role in the cell walls,[3, 4] namely moderate hydrophobicity, water-resistance and bactericidal and anti-biofouling properties. Significantly, similar results are not attainable in suberin samples isolated using any other method known so far, either chemical[1] or enzymatic,[5] putting emphasis on the role of cholinium hexanoate as a remarkable extraction solvent and catalyst. Overall, given the abovementioned facts, one

can recognise that this demonstration constitutes a significant scientific breakthrough and establishes the foundations towards the development of new bio-inspired polyester materials. Although, applications were not the aim of this thesis, given the myriad of versatile materials currently available, the use of such biopolyesters certainly requires further investigation. Evidently, preference should be given to possible uses that take advantage of the inherent antibacterial properties of suberin films. In line with the abovementioned results, on-going work in the Applied and Environmental Mycology Laboratory shows that the process herein presented can also be applied either for the extraction of suberin from potato peels either for the extraction of cutin, a suberin-like polyester, from multiple sources, *e.g.* tomato and apple peels. Importantly, these extracts showed also film forming ability enlarging dramatically the number of potential biopolyester film sources.

Emphasis should also be given to the fact that the partial preservation of suberin structure during ionic liquid-based depolymerisation makes these samples more similar to the native suberin, when compared to those attained by conventional methods. Therefore new insights into its *in situ* organisation may also be fostered. For instance, taking into account the preferential hydrolysis of acyl-glycerol ester bonds by cholinium hexanoate, one can reasonably estimate the role of glycerol in the overall cross-linking of the structure. Taking advantage of these features, this process was applied for suberin isolation in a time course analysis of *Solanum tuberosum* wound-healing. This work has been developed in collaboration with Professor Ruth Stark and her student Nancy Medina (CUNY, NY, USA) and the results are still under evaluation. Similarly, to further clarify the role of suberin as a protective barrier towards pathogens, suberin-fungal interactions are also being studied in the Applied and Environmental Mycology Laboratory. Moreover, this process constitutes an evidence of ionic liquid catalysed ester hydrolysis and may also provide new useful paths for triglyceride reactions. In this scope, ionic liquid tuneable properties open the possibility to design formulations displaying even higher selectivity towards a specific carbon centre of the acyl-glycerol ester bonds.

As in most extraction processes, performance improvements are mostly associated with modifications of the extraction solvent. Two bottlenecks hamper the cost-effective scale-up of this ionic liquid-based suberin depolymerisation process: the filtration step used to remove the insoluble residue and the ionic liquid recyclability.

After suberin dissolution in the ionic liquid, the insoluble cork residue is removed by filtration. However, due to the inherent high viscosity of the extraction media, filters clog quite rapidly. Dimethyl sulfoxide has been added as a co-solvent in order to decrease the viscosity of the media while keeping either the ionic liquid either suberin in solution. Importantly, preliminary scale-up tests in a 5 L tank suggested that the use of the co-solvent can be avoided when performing the filtration under pressure and at high temperatures. Cross-flow filtration may also show positive results regarding the clogging of the filters.

Residual yet cumulative degradation of the cholinium hexanoate over the extraction cycles is likely to occur. About 95 to 98 wt% of the ionic liquid can be recovered at the end of the first cycle as determined gravimetrically. Gas chromatography – mass spectrometry data of control tests confirmed the presence of ionic liquid degradation products (*e.g.* dimethylethanolamine and acyl glycerol esters containing transesterified hexanoate chains) in up to 3 wt% after four hours of reaction. In addition, since the current method to recover suberin relies on the addition of water, ionic liquid recyclability requires an energy-demanding evaporation step. In accordance, more cost-effective methods are desirable or alternatively, the use of other techniques such as azeotropic distillation should be considered. After recovery of the cholinium hexanoate, minor aliphatic impurities are still detected by nuclear magnetic resonance and mass spectrometry. These impurities are likely to accumulate in the ionic liquid however their impact in the overall efficiency is most possibly residual.

The influence of water content in the reaction media must be further investigated since water may play dual roles, *i.e.* depending on the amount it may be consumed during suberin hydrolysis or act as antisolvent for suberin precipitation. Similarly to other reports of biomass dissolution[6] this is one of the most difficult variables to control given its presence in biomass and the hygroscopic nature of cholinium hexanoate. Since suberin

dissolution has been related to the ionic liquid basicity in the same way as its hydrophilicity,[6, 7] there is no straight-forward approach to address this problem through ionic liquid design.

Given the abovementioned facts, and taking advantage of ionic liquids tuneable design, new formulations can be prepared with the aim of retaining the desirable features of the archetypal cholinium hexanoate. New formulations should preserve the hydrogen bond acceptor ability, the low toxicity and the high biodegradability, whilst lower viscosity, lower melting point, improved thermal and chemical stability are highly recommendable. The ionic liquid design should take into account the active catalytic role of both ions during ester bond hydrolysis, as demonstrated in Chapter V. Since suberin is largely composed of oxygen functionalised aliphatic chains, the ionic liquid should also comprise a somehow amphiphilic nature. In fact, the presence of long alkyl side chains in the cholinium carboxylate anion was proved to have a positive effect on the suberin extraction yield (Chapter II and III). This behaviour is likely attributed to the appropriate accommodation of suberin aliphatic moieties in the non-polar environment of the ionic liquid.

2. Microwave assisted extraction of betulin

The data herein presented regarding the microwave assisted extraction of betulin from birch outer bark are still preliminary. Nevertheless, this straight-forward process constitutes a very elegant advance in the isolation of highly pure betulin extracts. Significantly, great energy and time savings can be readily attained with this method when compared to conventional Soxhlet extractions. Also, both the solvent and the betulin source are renewable materials, an aspect that perfectly meets the Principles of Green Chemistry.[8] The major issue hampering the greenness of the process is certainly the use of cyclohexane as an antisolvent for betulin precipitation. Nevertheless, given the very low solubility of this triterpenoid in most organic solvents this seems an easily improvable task. Further work is envisaged, namely the efficient precipitation of betulin, limonene recycling and the optimisation of the operation conditions. One can reasonably

speculate that similar selectivity and performances can be attained in the extraction of other plant triterpenoids using terpenic solvents.

3. Greenness of the processes

The integration of both alternative methods herein presented, *i.e.* extraction of triterpenes and of suberin, may greatly stimulate the perceived value of cork and birch outer bark residues. This strategy perfectly fits the biorefinery concept. Much should be done to advance in this fascinating field, yet both methods illustrate that natural products can be selectively extracted from complex matrixes through rational selection of the solvents and systematic planning of the processes. Despite still in early stages of development, suberin mild depolymerisation with cholinium hexanoate and microwave assisted extraction of betulin are here preliminarily evaluated in respect to the 12 Principles of Green Chemistry[8] (Table 1). In the first method, environmental acceptability is mostly hampered by the ionic liquid stability and by its energy consuming recyclability. Such challenging issues are not identified in the microwave assisted extraction of betulin, where the greenness of the process is mainly impaired by the used antisolvent. This issue seems however to be easily improvable.

Despite some redundancy a final comment on the use of ionic liquids as solvents for biomass dissolution needs to be added. Similarly to that observed for the isolation of suberin with cholinium hexanoate, the aforementioned difficulties occur across the field, even in those cases that are pointed out as the most successful ones, such as the dissolution of cellulose. It is undeniable that ionic liquids provide new solutions for the fulfilment of the green chemistry and biorefinery concepts regarding biomass processing nevertheless many challenges are envisaged, mostly arising from the ionic liquid. Thermal and chemical stability of ionic liquids under process conditions has been often overestimated. Formulations showing strong hydrogen bond basicity (incidentally those ones more appropriated for biomass dissolution) undergo thermal degradation, even at moderate temperatures due to their higher reactivity.[6, 9] This constitutes a critical aspect for process sustainability and cost-efficiency and consequently alternative ionic liquid formulations should be envisaged. The study of water content effect, ionic liquid cost-

Table 1| Evaluation of suberin mild depolymerisation with cholinium hexanoate and microwave assisted extraction of betulin in respect to the 12 Principles of Green Chemistry.

	Suberin mild depolymerisation with cholinium hexanoate	**Microwave assisted extraction of betulin**
1.	*Prevention – it is better to prevent waste than to treat or clean up waster after it is formed.*	
	- The "desuberised" cork and birch outer bark insoluble residues constitute the wastes of the process. These are biomass residues and pose no risks. - Minor amounts of by-products arise from ionic liquid degradation and side reactions (transesterification). As a result ionic liquid cannot be continuously re-used.	- The extractive-free birch outer bark constitutes the wastes of the process. This is a biomass residue and poses no risks. - No side reactions are observed. - Despite further evaluation is required, data suggests that limonene can be recycled and re-used.
2.	*Atom economy – synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product. (Atom economy = 100 · MW products / MW reactants; MW, molecular weight)*	
	- Considering suberin and water as the only reactants, the atom economy of the process is 100%.	- n.a. (no reaction occurs)
3.	*Less hazardous chemical synthesis – wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.*	
4.	*Design safer chemicals – chemical products should be designed to affect their desired function, while minimizing their toxicity.*	
5.	*Safer solvents and auxiliaries – the use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.*	
	- Cholinium hexanoate is regarded as a benign solvent (see Green Chemistry Principle 10). Even though further lifecycle assessment studies should be considered. - The use of DMSO during insoluble residue filtration should is detrimental for the environmental sustainability of the process. Importantly, its use can be avoided by proper equipment design. - The use of water as an antisolvent does not pose risks. However, it raises technical challenges concerning its evaporation for ionic liquid recovery (Green Chemistry Principle 6). - Cork, birch outer bark and suberin does not pose risks for human health or environment.	- Limonene is generally regarded as a safe chemical. However, in bulk quantities, limonene is flammable, irritant after long term exposure and very toxic to aquatic organisms. Despite, these adverse properties, the selectivity of the process relies on its use. - The antisolvents, cyclohexane and ethyl formate, are flammable, irritant and harmful for human health and environment. They might be substituted by other less hazardous compound. - Birch outer bark does not pose any risks for human health and environment. As a natural triterpenoid, no severe risks are expected to be raised by betulin.
6.	*Design for energy efficiency – energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.*	
	- Processing of suberin in cholinium hexanoate is performed at 100 °C during 4 hours. The time and temperature required for this process makes it an energy intensive process. - Ionic liquid recovery relies in water evaporation, an energy demanding process.	- Microwave assisted extraction of suberin allows considerable energy savings. Microwaves can induce rapid and efficient heating with low energy consumption. In addition, the short period necessary for complete betulin extraction further highlights energetic performance of this process. - The energy required for limonene recovery through antisolvent evaporation depends on the choice of this compound. Currently, cyclohexane and ethyl formate have been used.

Table 1| (continued)

7. *Use of renewable feedstocks – a raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.*	- Cork and birch outer bark are plant residues. - Cholinium hexanoate incorporates biodegradable and non-hazard ions, however its synthesis involves several petroleum-based chemicals, such as cholinium chloride.	- Birch outer bark is a plant residue. - Limonene can be obtained from natural sources, even though it may pose some risks when used in bulk quantities.
8. *Reduce derivatives – unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.*	- n.a.	- n.a.
9. *Catalysis – catalytic reagents (as selective as possible) are superior to stoichiometric reagents.*	- Cholinium hexanoate acts simultaneously as a solvent and a selective catalyst. It was proved that similar behaviour cannot be attained when using common solvents and catalysts.	- n.a. (no reaction occurs)
10. *Design for degradation – chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.*	- Biomass residues and suberin are inherently biodegradable and biocompatible.[10, 11] - Cholinium hexanoate incorporates biodegradable and non-hazard ions, being therefore generally regarded as fully biodegradable.[12, 13] It shows reduced toxicity towards filamentous fungi,[12] even though further lifecycle assessment studies should be considered. Cholinium chloride, classified as a provitamin in Europe and widely used as animal feed supplement, is synthesised in a one-step solvent-free reaction of hydrogen chloride, ethylene oxide and trimethylamine with an atom efficiency of 100%.[14, 15]	- In its natural lifecycle birch outer bark is mineralised in soil. Therefore betulin environmental persistence is not expected. The same rational may be applied to limonene.
11. *Real-time analysis for pollution prevention – analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.*	- n.a.	- n.a.
12. *Inherently safer chemistry for accident prevention – substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.*	- No hazards arise from the use of cholinium hexanoate, cork or birch outer bark under process conditions.	- No significant hazards arise from the microwave assisted extraction. This technique promotes a rapid heating of the extraction media. Yet, since limonene has a high boiling point (176 °C), no internal pressure is created under process conditions. - The use of bulk quantities of flammable and hazard solvents constitutes the main risk source regarding environmental and human safety.

n.a. – not applicable.

efficient recyclability and the accurate evaluation of the ionic liquid environmental fate are also highly recommendable. Finally, one should note that a huge endeavour has been placed in imidazolium and pyridinium families of ionic liquids, while other more benign formulations have been neglected. The same rational applies to cellulose relatively to other less abundant biopolymers.

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No final, talvez o mais importante não seja o objectivo em si, mas sim, aquilo em que nos tornamos para o alcançar.

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